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# NMR Study of Neurophysin Dimer Dissociation by Cosolvents

Jian Yao

*Portland State University*

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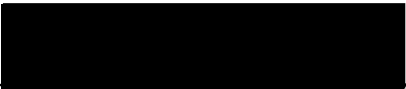
The abstract and thesis of Jian Yao for the Master of Science degree in Chemistry were presented April 29, 1996 and accepted by the thesis committee and the department.

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## ABSTRACT

An abstract of the thesis of Jian Yao for the Master of Science in Chemistry presented April 29, 1996.

Title: NMR Study of Neurophysin Dimer Dissociation By Cosolvents

Neurophysins (NPs) make up a relatively small, stable, and highly soluble class of proteins. They have physiological roles of storage and stabilizing of peptide hormones oxytocin and vasopressin within the posterior pituitary neurosecretory granules. At the concentration of NP found within the granules, NP would exist as a dimer in the absence or presence of bound peptide. The NP monomer-monomer interface involves  $\beta$ -sheet/ $\beta$ -sheet contact, which can be modulated by the presence of cosolvent. This remarkable feature of NP makes it a model for Alzheimer's disease. One of the characteristics of Alzheimer's disease is the presence of plaques of  $\beta$ -amyloid protein that are deposited on the brain. The plaques are rich in  $\beta$ -structure. Being water-insoluble makes them impossible to be directly studied by solution-state NMR.

The purpose of this study was to modify the solvent system to lower the NP dimerization constant and characterize the nature of solvent on

dissociation of dimer. A set of cosolvents was selected to try to reduce NP dimerization at relatively high concentration of NP. The organic cosolvents included deuterated methanol, dimethyl sulfoxide, ethyl acetate, propionitrile, and acetonitrile. Also, the protein unfolding reagents, deuterated urea and guanidine monohydrochloride, were tried. The interaction between bromophenol blue and NP was also studied because this dye binds predominately to the dimer form of NP. High-resolution NMR techniques were used to sense the NP-I dimer/monomer equilibrium.

Among the organic cosolvents used, only acetonitrile and propionitrile were found shift the dimer  $\rightleftharpoons$  monomer equilibrium significantly toward monomer. The cosolvent probably changed the character of the solvent system, penetrated the monomer-monomer interface and interacted with the interface residues, caused the break up of dimer. The unfolding reagents were found to partly unfold the NP simultaneously with dissociation of the dimer. Bromophenol blue binds to NP-I at low pH, but the solubility of NP-dye complex is too low to be studied extensively by solution-state NMR methods.

NMR STUDY OF NEUROPHYSIN DIMER DISSOCIATION BY  
COSOLVENTS

by

Jian Yao

A thesis submitted in partial fulfillment of the requirement for the degree  
of

MASTER OF SCIENCE  
in  
CHEMISTRY

Portland State University

1996

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## TABLE OF CONTENTS

|  | PAGE |
|--|------|
| ACKNOWLEDGMENTS .....                      | ii   |
| LIST OF TABLES .....                       | iv   |
| LIST OF FIGURES .....                      | v    |
| CHAPTER                                    |      |
| 1 GENERAL INTRODUCTION .....               | 1    |
| Overview of Literature .....               | 1    |
| Overview of This Work .....                | 8    |
| 2 EXPERIMENTAL METHODS AND MATERIALS ..... | 14   |
| Protein Preparation .....                  | 14   |
| NMR Experiments .....                      | 18   |
| 3 RESULTS AND DISCUSSION .....             | 24   |
| Organic Cosolvents .....                   | 24   |
| Denature Reagents .....                    | 39   |
| Dye .....                                  | 43   |
| 4 CONCLUSION .....                         | 65   |
| REFERENCES .....                           | 67   |
| APPENDIX .....                             | 72   |

## LIST OF TABLES

TABLE

PAGE

|    |   |    |
|----|---|----|
| I  | Physical properties of cosolvent and its ability to break dimer ..... | 35 |
| II | List of dimer/ monomer sensitive resonances .....                     | 36 |



## LIST OF FIGURES

| FIGURE   | PAGE |
|--|------|
| 1. The x-ray Crystal structure of NP-II.....   | 9    |
| 2. Primary sequence of NP-I .....  | 10   |
| 3. Aliphatic (A), and aromatic (B) proton resonances of native NP-I .....                          | 11   |
| 4. Effect of des-1-8 NP-I concentration at 6.44 and 6.2 ppm .....                                  | 12   |
| 5. 1D NMR spectra of acetonitrile titration of NP-I .....  | 13   |
| 6. Column chromatography for isolation of NP-I .....   | 22   |
| 7. Pulsed 1D NMR measurement (A), and Example of pulsed 2D NMR (B) .                               | 23   |
| 8. Spectra of deuterated cosolvents in D <sub>2</sub> O .....                                      | 45   |
| 9. 1D NMR spectra of propionitrile titration of NP-I .....   | 46   |
| 10. Aliphatic region spectra of propionitrile titration of NP-I .....                              | 47   |
| 11. Aromatic region spectra of propionitrile titration of NP-I .....                               | 48   |
| 12. 1D spectra of methanol titration of NP-I .....   | 49   |
| 13. 1D spectra of dimethyl sulfoxide titration of NP-I .....                                       | 50   |
| 14. 1D spectra of ethyl acetate titration of NP-I .....  | 51   |
| 15. Relation between physical properties of cosolvent and its ability to<br>dissociate dimer ..... | 52   |
| 16. 2D NOESY spectra of 1 mM NP-I without acetonitrile .....                                       | 53   |
| 17. 2D NOESY spectra of NP-I with 10% acetonitrile-d <sub>3</sub> .....                            | 54   |
| 18. 2D NOESY spectra of NP-I with 10% acetonitrile .....   | 55   |
| 19. 1D spectra of guanidine monohydrochloride .....  | 56   |

|   |    |
|---|----|
| 20. 1D spectra of guanidine DCl titration of NP-I .....                       | 57 |
| 21. 2D NOESY spectra of NP-I (A), and NP-I in 7 M Gnd·DCl (B) .....           | 58 |
| 22. 2D ROESY of NP-I in 2.5 M Gnd·DCl .....                                   | 59 |
| 23. 1D Spectra of urea in D <sub>2</sub> O .....                              | 60 |
| 24. 1D spectra of urea titration of NP-I in D <sub>2</sub> O .....            | 61 |
| 25. 1D spectrum of bromophenol blue .....                                     | 62 |
| 26. 1D spectra of bromophenol blue titration of NP-I .....                    | 63 |
| 27. 1D spectra of bromophenol blue titration of NP at low concentration ..... | 64 |

# CHAPTER 1

## GENERAL INTRODUCTION

### Overview of Literature

#### Significance

Neurophysin (NP) is co-synthesized in vivo with peptide hormones oxytocin and vasopressin, and has physiological roles which include storage and stabilizing of these peptide hormones within the posterior pituitary neurosecretory granules (Breslow and Burman, 1990). Oxytocin is known to mediate uterine contraction and milk ejection, and plays an important role in sexual behavior and response, as well as the bonding between parent and offspring. Vasopressin plays an important role in influencing kidney function, blood pressure and body fluids. Both hormones are nonapeptides and are found in concentrations as high as about 0.1 M in the neurosecretory granules of the posterior pituitary (Dreifuss, 1975), complexed in a 1:1 molar ratio with neurophysins. During axonal transport the linking residues (Gly-Lys-Arg) between the hormone and NP are cleaved, leaving the hormone bound to NP *via* non-covalent interactions. These interactions are important for the stabilization of the hormone within the granules (Rholam et al, 1981). When demand for the hormone is received, it is released into the blood stream as the NP- hormone complex, which then dissociates to give the free hormone and NP.

In addition to its biological significance, the NP- hormone system has been regarded as a very useful model for understanding protein-peptide interactions (Barbar, 1992). Solution studies of NP using nuclear magnetic resonance spectroscopy (NMR) and other biophysical and biochemical methods have led to an accumulation of considerable data pertaining to protein-peptide interaction for the NP-hormone system and NP dimerization (Breslow and Burman, 1990). The thermodynamical linkage between NP-hormone interaction and NP self association is another reason that the NPs are interesting molecules to study. At the biological conditions in the pituitary, NPs self associate to dimer. The monomer-monomer interface is comprised mainly of  $\beta$ -sheet structures. This self-association forms a model for intermolecular association involving  $\beta$ -structure.

### Introduction of NP

NPs are particularly sulfur-rich proteins. The fourteen Cys residues per polypeptide chain are paired as seven disulfide bridges. These confer a high degree of stability to NP under conditions compatible with disulfide integrity. NPs have a very low content of aromatic amino acids, but a high proportion of proline. The presence of a large number of acidic residues results in a low isoelectric point pI of 4.5-5.5 (Pickering and Jones, 1978).

Two major classes of bovine neurophysins, denoted NP-I and NP-II are known (Hollenberg and Hope, 1968). They have very similar physical and chemical properties, differing slightly in amino acid composition. Each has a molecular mass of about 10,000 Dalton. Circular dichroism and binding properties of the two are almost identical (Breslow and Burman, 1990).

Ultracentrifuge studies show that there is no species higher than dimer in solution at the concentrations used for NMR (Nicolas and Batelier, 1980), and the dimer  $\rightleftharpoons$  monomer equilibrium shifts to dimer as the pH decreases (Carlson and Breslow, 1981). As evidenced by CD results, no large secondary structure changes occur upon dimerization, but there are subtle changes in tertiary structure that involve the binding site for the ligand oxytocin or vasopression. These changes play a potential role in the stronger binding of peptide by dimer than by monomer (Breslow et al., 1992). The monomer-monomer interface is comprised of  $\beta$ -sheet interactions involving residues 32-38 and 77-81 (Chen et al., 1991). Tyr-49 and Thr-9 are perturbed by dimerization as monitored by NMR (Peyton et al., 1986; Breslow et al., 1992), although they are distant from the monomer-monomer interface in the crystalline liganded state (Chen et al., 1991). Dimerization induced at least two distinct slowly exchanging environmental states for the 3,5 ring protons of Try-49. In the monomer, it is likely that there is a rapid equilibrium between multiple forms (Breslow et al., 1992). Two environments were also found in the dimer of des-1-8 NP-I for the methyl protons of Thr-9 (Breslow et al., 1992).

NMR was used in monitoring the NP's dimer and monomer equilibrium shift by dilution of NP (Peyton et al., 1986), as well as in NP assignment and the acetonitrile cosolvent effect on NP (Barbar, 1992). Also, NMR has been used for many other proteins that involve dissociation of dimer to monomer (e.g. Johnson et al., 1995; Gitelson et al., 1991). Thus NMR is a good tool to sense the shift in NP dimer  $\rightleftharpoons$  monomer equilibrium.

As a reference to this study, the crystal structure of NP-II hormone complex (Chen et al., 1991), which is very similar to NP-I, is shown in Figure 1. As shown in Figure 1A, the structure of each chain is 12% helix and 40%  $\beta$ -sheet,

and the chain is folded into two domains. The amino-terminal domain begins with a long loop (residues 1-10), then enters a four-stranded antiparallel  $\beta$ -sheet (residues 11-13, 19-23, 25-29, and 32-37), followed by a 3-turn  $3_{10}$ -helix (residues 39-49) and another loop (residues 50-58). The carboxyl-terminal domain is shorter, consisting of only a four-stranded antiparallel  $\beta$ -sheet (residues 59-61, 65-69, 71-75, and 78-82). Figure 1B shows a drawing of the C $\alpha$  backbone, including monomer-monomer contacts that involve two layers of eight antiparallel  $\beta$ -sheets. Three known monomer-monomer interface residues in NP-I, Phe-35, Thr-38 and His-80, are indicated. The primary sequence of NP-I (Pickering, 1978) is also included (Figure 2). The 3-turn  $3_{10}$ -helix region is in bold face, and antiparallel  $\beta$ -sheet regions are underlined.

### Concentration Sensitivity

At the concentrations of NP found within the granules, NP would exist as a dimer in the absence or presence of bound peptide (Breslow and Burman, 1990). In the absence of bound peptide, NP exists in monomer  $\rightleftharpoons$  dimer equilibrium, with dimerization constants in the range of  $5 \times 10^3$  to  $10^4 \text{ M}^{-1}$  at neutral pH (Nicolas and Batelier, 1980). A broad range of NP concentrations has been used in NMR investigations, which permitted spectra of the native protein to be obtained under conditions ranging from essentially pure monomer to pure dimer (Peyton et al., 1986). Figure 3 shows the aliphatic and aromatic proton NMR spectra for native NP-I at concentrations of 0.02 mM, 0.2 mM, and 3 mM at pH 6.2. Arrows point to concentration-sensitive resonances. Since most of the concentration-dependent resonances could not be assigned unambiguously, they were used largely as empirical indicators of dissociation of dimer. In the upfield region around 0 to 1 ppm, resonances shift upfield

with an increasing concentration of NP-I. Between 1.1-1.4 ppm region, the peaks are more resolved in the low-concentration spectrum. Around region 1.7 ppm, there is a resolved resonance from the dimer. At the 2.5 ppm region, resonances are more resolved in monomer. There is a relatively sharp tip at 3.8 ppm in the high-concentration spectrum.

In the aromatic proton region of the native protein, Figure 3B, four concentration-dependent changes are clearly seen at pH 6. With increasing concentration, there are marked broadening of the unambiguously assigned Tyr-49 3,5 ring protons peak at 6.79 ppm (Balaram et al., 1973) and 2,6 ring protons at 7.1 ppm (Peyton et al., 1986). There is also the disappearance of a sharp peak at 7.22 ppm at high concentration relative to low concentration, as well as increased resolution of a peak at 7.58 ppm. These two peaks were previously assigned to Phe (sum of Phe-35 and Phe-22) and Phe-35, respectively (Breslow et al., 1995, and Lord et al., 1980).

As seen in Figure 4, there are concentration-dependent peaks at 6.44 ppm and 6.2 ppm. The 6.44 ppm peak increases with increasing concentration of NP-I to a limiting intensity representing one proton, while at 6.2 ppm decreases in intensity with increased concentration. The 6.44 and 6.2 ppm peaks were assigned to dimer and monomer, respectively (Breslow et al., 1992). The ratio of these intensities as a function of concentration correlates well with known dimerization constants (e.g., see Nicolas et al., 1980, and Peyton et al., 1986). The degree of dimerization, as evidenced by the intensity of the 6.4 ppm peak, also correlates with the loss of sharp Tyr 3,5 ring protons signal assignable to monomer (Breslow et al., 1992).

### Acetonitrile Cosolvent Sensitivity

In a previous study, it was found that the NP-I proton NMR spectrum was sensitive to the amount of acetonitrile in a D<sub>2</sub>O/ acetonitrile solvent mixture (Barbar, 1992). As a cosolvent, acetonitrile was found lower the dimerization constant of NP, and decrease the solvent viscosity without causing a major conformation change of protein (Barbar, 1992).

Figure 5 shows a set of 1 D spectra of NP-I acquired with an increasing volume percent of acetonitrile. The increase in spectral resolution was manifested by narrowed peaks and increased resolution in the  $\beta$ -sheet region of the H $\alpha$  resonances, which is between 4.8 and 6.5 ppm (Barbar, 1992). Also, the increase in resolution is an indication of the formation of monomer in D<sub>2</sub>O/ acetonitrile.

In the upfield region, between 0 and 1 ppm, there are resonances around 0.6 and 0.4 ppm. Upon dissociation of dimer, the resonances shift downfield. Like what happens in the concentration-dependent spectra, between 1.1-1.4 ppm region, a resonance at 1.25 ppm is a broad shoulder in the dimer. This resonance becomes sharper with increasing concentration of acetonitrile. The resonance at 1.3 ppm is a single peak under dimer condition, it becomes multiple peaks under monomer condition. Around 1.7 ppm, there is a resolved resonance in the dimer, but it merges with a big peak upon dissociation in the presence of acetonitrile. As also observed in the 2.5 ppm region in concentration-dependent spectra, resonances are more resolved in monomer.

For Tyr-49 of NP-I, signals at about 6.7 and 7.1 ppm in the dimer (3,5 and 2,6 ring protons, receptively) get sharper upon dissociation of dimer to monomer at high concentration of acetonitrile. Also, the dissociation of dimer is



accompanied by a shift of the peak at 7.57 ppm and an increasing sharp tip at 7.2 ppm, which were assigned to Phe-35 and to Phe (sum of Phe-35 and Phe-22) (Breslow et al., 1995). As in the concentration-dependent spectra, the dimer peak at 6.4 ppm, lost its intensity upon increasing acetonitrile concentration, while the monomer peak at 6.1 ppm increases in intensity and shifts downfield. A resonance at 8.4 ppm, which was assigned to His-80 C<sub>2</sub>H, gets sharper at high acetonitrile concentrations (Breslow et al., 1992).

Thus, the dimerization-sensitive resonances found in concentration-dependent spectra are generally consistent with those found in cosolvent-dependent spectra. The changes of these sensitive resonances are specific effects of dimerization on molecular structure and, for the most part, do not represent nonspecific effects such as viscosity-induced broadening (Peyton et al., 1986). Addition of cosolvent and lowering the NP concentration have the same effect on NP, shifting the neurophysin monomer  $\rightleftharpoons$  dimer equilibrium toward monomer.

Do NPs only dissociate in acetonitrile? Do any other solvent systems have the same effect? The answers to these questions might provide clues about the mechanisms of NP dissociation. So a set of cosolvents were studied to see if they break the NP dimer.

## Overview of This Work

NP's self-association can be modulated by solvent system. Therefore, a set of cosolvents was titrated into the regular deuterated solvent ( $D_2O$ ). The dimer-dissociation-sensitive resonances were monitored by NMR spectroscopy. It was found that the NP spectrum has different sensibility to different cosolvent. Among the organic cosolvents used, only acetonitrile and propionitrile be able to lower the NP dimerization constant significantly, presumably because they disturbed the hydrophobic monomer-monomer interaction and destabilized the  $\beta$ -sheet hydrogen bond contact. The interaction between acetonitrile and monomer-monomer interface was further studied by 2 D NOESY spectra. From the observations, it was concluded that acetonitrile may interact with interface residues to cause dissociation of dimer. By using the protein denaturation reagents guanidine·DCl and urea, it was found that NP did not dissociate until it was unfolded. The binding of bromophenol blue to NP was also examined. The NP-dye complex was formed, although it was not soluble enough to be studied by NMR.

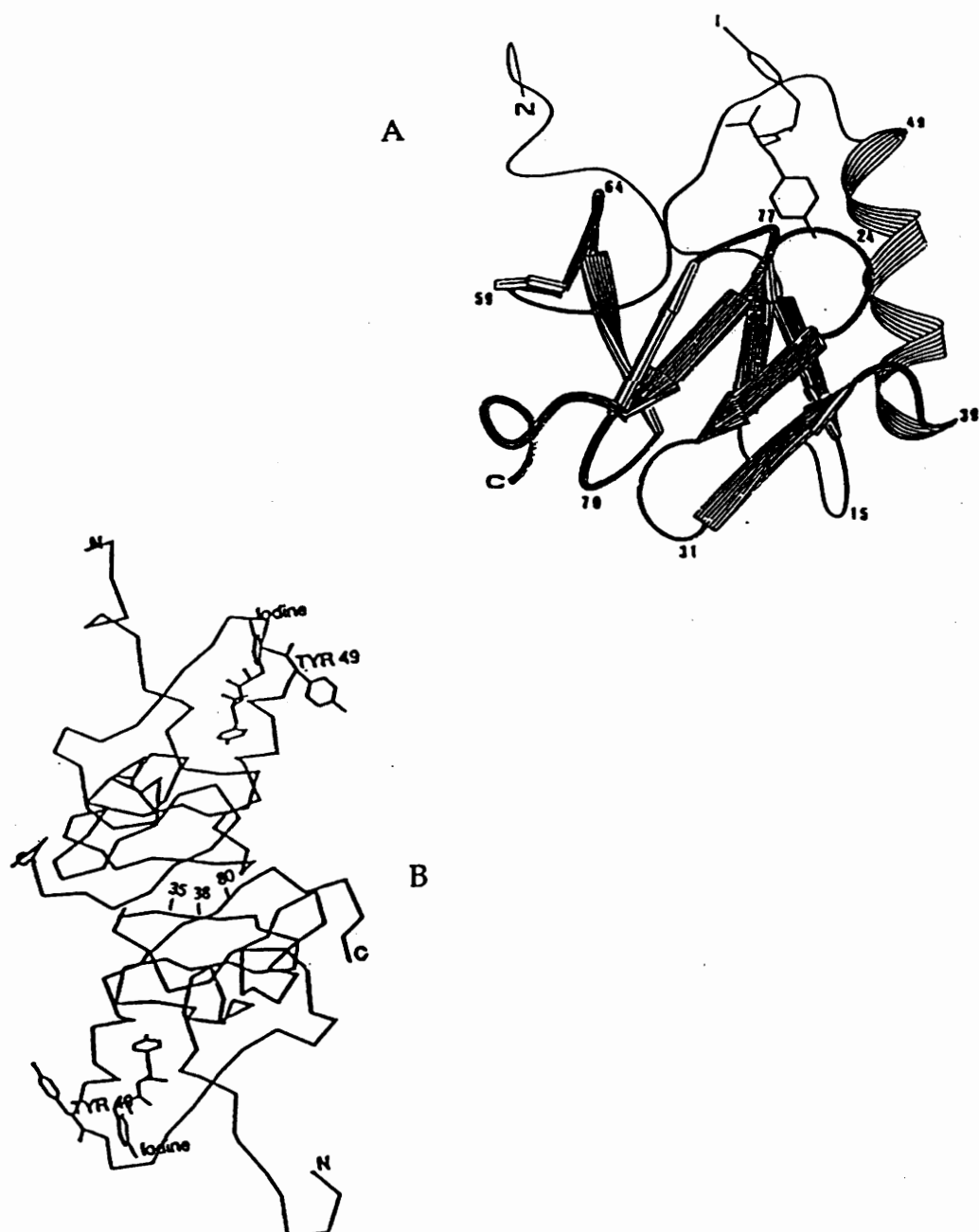


Figure 1: The x-ray crystal structure of NP-II bound to a dipeptide hormone analog. (A) A schematic representation showing that the protein is composed of two domains, each with four antiparallel  $\beta$ -sheets. The two domains are connected by a helix followed by a long loop. (B) Stereo drawing of monomer-monomer contacts involving antiparallel  $\beta$ -sheet interactions. Three known monomer-monomer interface residues, Phe-35, Thr-38 and His-80, are indicated. This figure is adapted from Chen et al., 1991.

Ala-Val-Leu-Asp-Leu-Asp-Val-Arg8-Thr-Cys-Leu-Pro-Cys-Gly-Pro-Gly-Gly-  
Lys-Gly19-Arg-Cys-Phe-Gly23-Pro-Ser-Ile-Cys-Cys-Gly29-Asp-Glu-Leu32-  
Gly-Cys-Phe-Val-Gly 37-Thr-Ala-Glu-Ala-Leu-Arg-Cys-Gln-Glu-Glu-Asn-  
Tyr49-Leu-Pro-Ser-Pro-Cys-Gln-Ser-Gly-Gln-Lys59-Pro-Cys-Gly-Ser-Gly-  
Gly65-Arg-Cys-Ala-Ala-Gly-Ile-Cys-Cys-Ser75-Pro-Asp-Gly78-Cys-His-Glu-  
Asp82-Pro-Ala-Cys-Asp-Pro-Glu-Ala-Ala91-Phe-Ser

Figure 2: Primary sequence of NP-I. Residues 39-49 which comprise a 3-turn 3<sub>10</sub>-helix are in bold face. Residues in anti-parallel  $\beta$ -sheet are underlined.

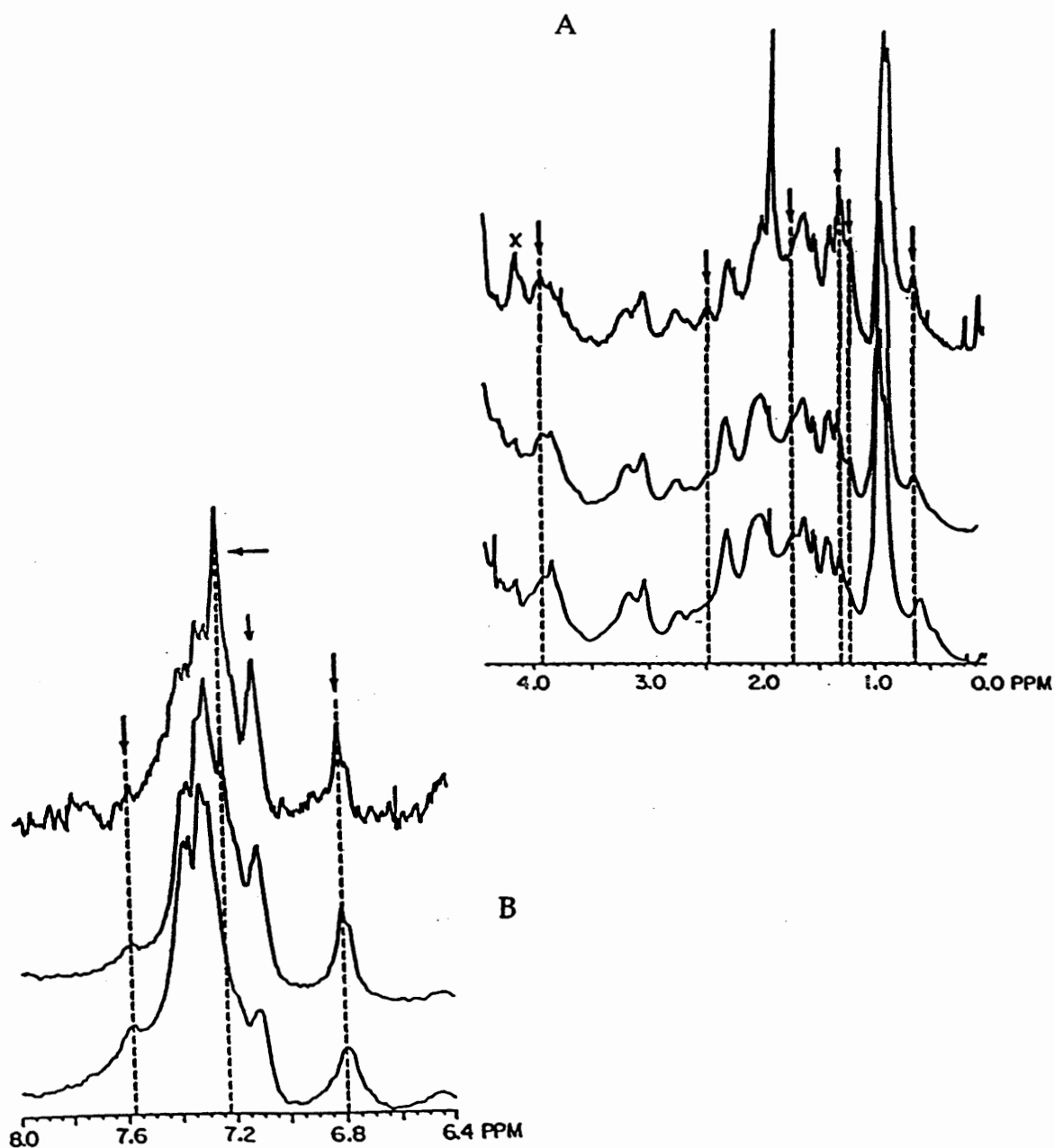


Figure 3: Aliphatic (A), and aromatic (B) proton resonances of native NP-I at pH 6.2 as a function of protein concentration. NP-I concentration from top to bottom: 0.02 mM, 0.1 mM, and 3 mM. Arrows point to concentration-sensitive resonances. The peak at 1.9 ppm is acetate. The peak marked by X is an artifact. The spectra were recorded at 300 MHz. This figure is adapted from Peyton et al., 1986.



Figure 4: Effect of des-1-8 NP-I concentration at 6.44 and 6.2 ppm, pH 6. Signals at 6.44 ppm and 6.2 ppm assigned to dimer and monomer, respectively. des-1-8 NP-I concentration from top to bottom: 1 mM, 0.25 mM, and 0.05 mM. The reason why the 6.8 ppm peak looks different from Figure 3 is that this is des-1-8 NP-I, native NP-I with out residues 1 to 8. The spectra were recorded at 500 MHz. This figure is adapted from Breslow et al., 1992.

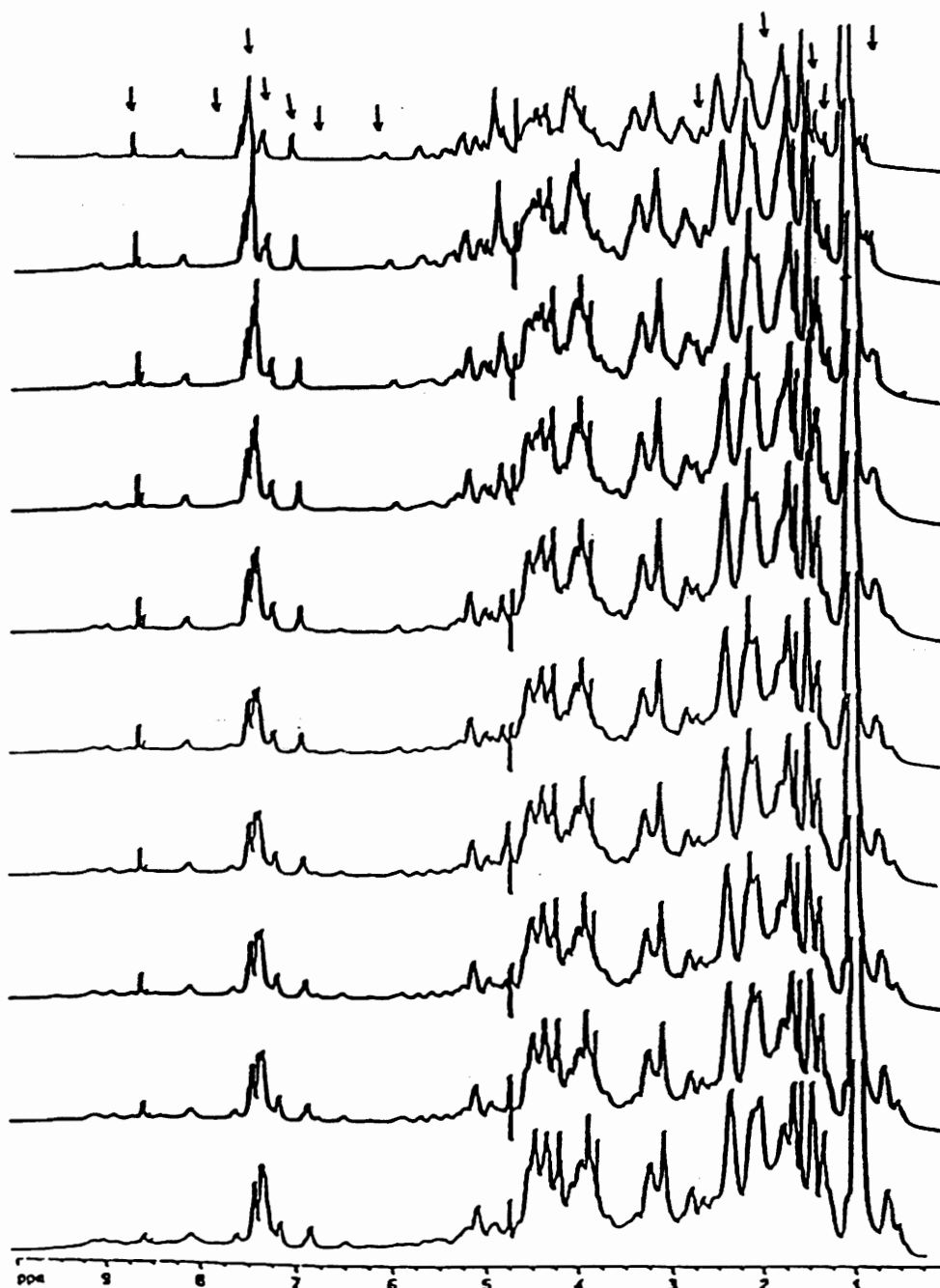


Figure 5: 1D NMR spectra showing acetonitrile titration of NP-I in D<sub>2</sub>O. Spectra were obtained at 35 °C, pH 6.0 in increasing concentration of acetonitrile from 0% (bottom), 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 25% to 30% (top). Arrows show the acetonitrile-sensitive resonances. This figure is adapted from Barbar, 1992.

## CHAPTER 2

### EXPERIMENTAL METHODS AND MATERIALS

#### Protein Preparation

Unliganded NP-I was used in this study, because the peptide-hormone ligand binding shifts the NP monomer-dimer equilibrium toward the dimer. The dimerization constant is thus increased by a factor variably estimated as 10 to 100 for liganded NP (Breslow and Garguilo, 1977).

Isolation and purification of NP-I from bovine pituitaries were done by a combination and modification of literature preparations (Whittaker and Allewell, 1984; Breslow et al., 1971; Hollenberg and Hope, 1968). The starting material for neurophysin preparation was fresh bovine pituitaries, which are commercially available from Pel-Freeze Biologicals. The fresh pituitaries were first lyophilized. Then the isolation of the crude neurophysin-hormone complex was done by extracting the lyophilized pituitaries with 0.1 N HCl, conditions under which proteolysis is minimal and the hormone-NP complex is soluble. The hormones were then separated from crude NP by chromatography on Sephadex G-75 in 1 N formic acid. The crude NP was next resolved into its individual fractions by chromatography on Sephadex-DEAE-50 in 60 mM, pH 7.9 Tris-HCl buffer using a NaCl gradient. NP was further purified by dialysis and chromatography over Sephadex G-25 in 0.1 N acetic acid.



A typical preparation was as follows: 33 g of fresh bovine pituitaries were lyophilized for 48 hours by using VIRTIS mode 10-020 lyophilizer. Then the lyophilized pituitaries (14 g) were extracted with 600 mL 0.1 N HCl. The mixture of the acid and pituitaries was ground with a blender and incubated at 4 °C for 20 hours. The final pH was 1.63. Insoluble material was removed by centrifugation at 27,300 x G ( $G = 32 \text{ feet/sec}^2$ ) with a GSA rotor for 1 hour at 0 °C using a SORVAL RC 2-B refrigerated centrifuge. The insoluble material was re-extracted with 400 mL 0.1 N HCl for another 20 hours at 4 °C. The final pH was 1.60. Supernatants were combined, then neutralized with about 50 mL of cold 2 N NaOH. Care was taken not to raise the pH above 7.0 to minimize proteolytic activity. The coffee-colored cloudy solution was centrifuged at 27,300 x G with a GSA rotor for 1 hour at 0 °C. The supernatant was decanted, and its pH adjusted to 3.90, followed by addition of 10 g of finely ground NaCl for every 100 mL solution to cause precipitation of the protein at 0 °C (Hollenberg and Hope, 1968). Precipitation was allowed to proceed for 20 hours at 4 °C, and then the suspension was centrifuged at 27,300 x G for 30 minutes. The sediment, which was the crude protein-hormone complex, was dispersed in 100 mL of water and dialyzed with Sepectrapor membrane tubing (MW cutoff = 6 KD) against water (4 x 4 L) to remove the salt. The small amount of residual precipitate was dissolved by the addition of several drops of 1 N acetic acid after the last dialysis, and the solution was then lyophilized. The protein-hormone complex weighted 600 mg.

Separation of the hormones from the crude NP-hormone complex was accomplished by dissolving half of the protein-hormone complex (300 mg), in 30 mL 1 N formic acid and chromatographing on a Sephadex G-75 column (6 x

60 cm), which has a useful working range of 3,000-70,000 D. The separation was conducted at room temperature, with 1 N formic acid as eluting solvent (Breslow et al, 1971). The column was connected to an ISCO UA-5 absorbance / fluorescence detector, set at 254 nm, equipped with a model 1133 Mutiplexer-Expander. Fractions were collected with a Retriever IV fraction collector. From the chromatogram shown in Figure 6A , three major fractions were resolved. The first two fractions represented high molecular weight proteins, which were colored brown. The third fraction was crude neurophysin, as was verified by NMR. The crude neurophysin fractions were lyophilized. This crude NP separation process was repeated for the other half of NP-hormone complex (300 mg). The total yield of crude NP was 75 mg.

The crude NP was fractionated at room temperature on a DEAE-Sephadex A-50 column (4 x 30 cm), which is a weak anion exchanger. The presence of several aspartic and glutamic acid side chains results in a low isoelectric point of 4.3 and 4.7 for NP-I and NP- II, respectively (Pliska et al., 1972). The column was pre-equilibrated with pH 7.90, 60 mM Tris-HCl buffer, and 75 mg of crude NP were placed on the column in 15 mL of the same buffer. Elution was performed with the same buffer, but using a linear 0 to 0.4 M NaCl concentration gradient. The elution flow was regulated by gravity. As shown in Figure 6B, four types of NP were obtained. The yield of the first peak after ion exchange, NP-II, was 11 mg. The second and third peak were NP-B and NP-C respectively. The last peak was NP-I, which was the protein used in this study. After dialysis against 0.1 N formic acid (4 x 4 L), the NP-I was further purified by Sephadex G-25 column (2.5 x 40 cm), which has a useful working range of 1000-5000 Daltons. This was done to remove last trace of salt and other impurities. The column was pre-equilibrated with 0.1 M acetic acid

buffer. The elution flow speed was controlled by a Wiz peristaltic pump. In Figure 6C, the first peak in G-25 spectra was purified NP-I. After lyophilized, NP-I weighed 13 mg. The proteins were stored at -20 °C.

The quality of the prepared NP-I and NP-II were checked by polyacrylamide gel electrophoresis (PAGE) by our collaborators, Dr. Breslow's research group. The results showed that both NP-I and NP-II were very clean. They were not contaminated by NP-B or NP-C or by each other.

## NMR Experiments

NMR is a powerful tool for analyzing neurophysin. With a chain length of approximately 95 residues, the monomer  $\rightleftharpoons$  dimer system is near the upper limit of molecular weight of protein typically accessible to detailed study by NMR spectroscopy. It was helpful that the  $^1\text{H}$  NMR spectrum of the NP-I was already partially assigned.

### NMR Sample Preparation:

All proton NMR studies were conducted at 25 °C unless otherwise stated. The samples were 0.4 - 0.5 mL in Wilmad 535pp NMR tubes which had been prerinsed twice with  $\text{D}_2\text{O}$ . Protein samples were typically prepared for NMR analysis by dissolving the lyophilized NP-I in 99.9%  $\text{D}_2\text{O}$ . The sample were then adjusted to the desired pD by using aliquots of dilute NaOD and/or DCl. Early studies showed no spectra changes resulted from the increased NaCl (Peyton et al., 1986).

The prepared NP-I NMR samples were titrated with the cosolvents, deuterated methanol (99.5% D), DMSO (99.9% D), propionitrile (99.7% D), and ethyl acetate (99.5% D). NP-I titration with deuterated urea, guanidine·DCl, and bromophenol blue were also done. The deuterated guanidine·DCl was prepared by dissolving guanidine·HCl in  $\text{D}_2\text{O}$ , and then lyophilizing repeated six times. The bromophenol blue was dissolved in  $\text{D}_2\text{O}$  to make a 0.03 M stock solution.

### 1 D NMR Experiments:

The procedure used to measure a conventional one-dimensional NMR spectra on a pulsed spectrometer is shown schematically in Figure 7A. During the preparation time a thermal equilibrium is established among the nuclear spins in the sample. The spins are then disturbed by a radio frequency (rf) pulse. The spin magnetization is monitored as its return to thermal equilibrium induces a signal that is detected by the receiver of NMR spectrometer. The time-dependent signal,  $s(t)$ , is known as the free induction decay (FID), and a Fourier transformation (FT) of the FID produces a frequency dependent spectrum,  $S(f)$ .

In this work, conventional 1 D  $^1\text{H}$  NMR experiments were run with the typical "zgpr" pulse sequence, RD-90°-FID, where 90° is the rf pulse of sufficient length ( typically about 8-14 ms) to give a maximal signal, and RD is relaxation delay. During the relaxation delay, the residual water resonance was suppressed with a low power, long pulse. The 1 D experiments were most often performed on a Nicolet NM 500 spectrometer modified with Tecmag Libra interface to a Macintosh IIfx. 1 D Fourier transform spectra were typically collected 4096 points over a spectral width of  $\pm 3000$  Hz. A total of 1024 transients were accumulated. A 0.3-Hz line broadening factor was applied, and then the FID was Fourier transformed to give the spectrum. All chemical shifts were referenced to TSP [sodium 3-(trimethylsilyl) propionate- $\text{d}_4$ ] through the water resonance at 4.76 ppm at 25 °C.

### 2 D NMR Experiments:

There are four periods in 2 D NMR experiments, preparation time, evolution time, mixing time, and detection time. An example 2 D experiment is

schematically shown in Figure 7B. As in 1 D measurements, the experiment starts with a preparation period to establish spin thermal equilibrium. The first pulse disturbs the spin system. After the pulse, the spin system evolves in the same way that it does in a 1 D measurement, although unlike 1 D experiments, the signal is not yet detected. There is mixing time separating the detection and evolution periods. The mixing time is designed to ensure that the information about the evolution of spin system during  $t_1$  is passed on to the detection period in a suitable form. So the evolution of the spin system again happens, and the signal during time  $t_2$  (measured from the end of the second pulse) of the detection period is detected and recorded. The FID [i.e., the dependence of the signal on time  $t_2$ ,  $s(t_2)$ ] also depends on the instant which the evolution of the spin system has been interrupted in the evolution period (i.e., on the duration of evolution period  $t_1$ ).

In a 2 D NMR experiment the length of evolution period  $t_1$  is varied in a systematic manner. For each value of  $t_1$ , the signal dependence on time  $t_2$  is acquired during the detection period. The entire set of these dependence for all values of  $t_1$  represents the function  $s(t_1, t_2)$ . A FT of this function with respect to both  $t_1$  and  $t_2$  produces a 2 D spectrum,  $S(f_1, f_2)$ .

Standard 2 D  $^1\text{H}$  NMR experiments used in this work were NOESY (Nuclear Overhauser Effect Spectroscopy) and ROESY (Rotating Frame Overhauser Effect Spectroscopy). Both of these rely on the NOE (Nuclear Overhauser Effect). The NOE involves dipole-dipole relaxation, which depends on the distant between the nuclei involved and effective correlation time of the vector that joins the nuclei, hence the molecular tumbling rate. For small molecules, the  $^1\text{H}$ - $^1\text{H}$  NOE is positive with a maximum intensity of 0.5. For intermediate size molecules, the NOE is small or zero. Large molecules have a negative

NOE with a maximum intensity of -1. 2 D NOESY observed in a rotating frame is called ROESY. The major advantage of doing ROESY is that all NOEs are positive regardless of molecular size in the rotating frame. In the case of chemical exchange in equilibrium systems, a negative cross peak can result, regardless of molecular size. Thus, the spin-locking experiment ROESY insures that the sign of NOEs is positive, while chemical exchange intensity is negative. 2 D NOE experiments NOESY and ROESY provide information about protons that are close in space (less than about 4.5 Å) and chemical exchange.

The pulse sequence used in this work was a modification of the Bruker noesyprtp, which we call "NOESYH2P11tp", RD-90°-t<sub>1</sub>-90°-t<sub>m</sub>-90°-x-τ-90°-x-FID(t<sub>2</sub>). The 90° pulse length was 9 μs, RD is the relaxation delay, which was 2.5 s, t<sub>1</sub> is the evolution time, t<sub>m</sub> is the mixing time, which was 150 ms, and t<sub>2</sub> is the acquisition time. The 2 D NMR experiments were performed on a Bruker AMX-400 spectrometer interfaced to Bruker X32 computer. During the delay time, a low power pulse was applied in some cases to saturate the residual water resonance. The spectral width is 16.02 ppm (6480 Hz). A total of 512 increments in t<sub>1</sub> was used. Data sets were acquired as 1024 points in the t<sub>2</sub> dimension and 700 points in t<sub>1</sub> dimension. The final data matrix was zero-filled to 1024 x 1024 real data points. ROESY was acquiesced in a similar way, except that the mixing time, t<sub>m</sub>, was done with a spin-locking field turned on. The pulse sequence was RD-90°-t<sub>1</sub>-90°-t<sub>m</sub>-(SL)-FID(t<sub>2</sub>), where SL is the lower pulse for the ROESY spin lock.

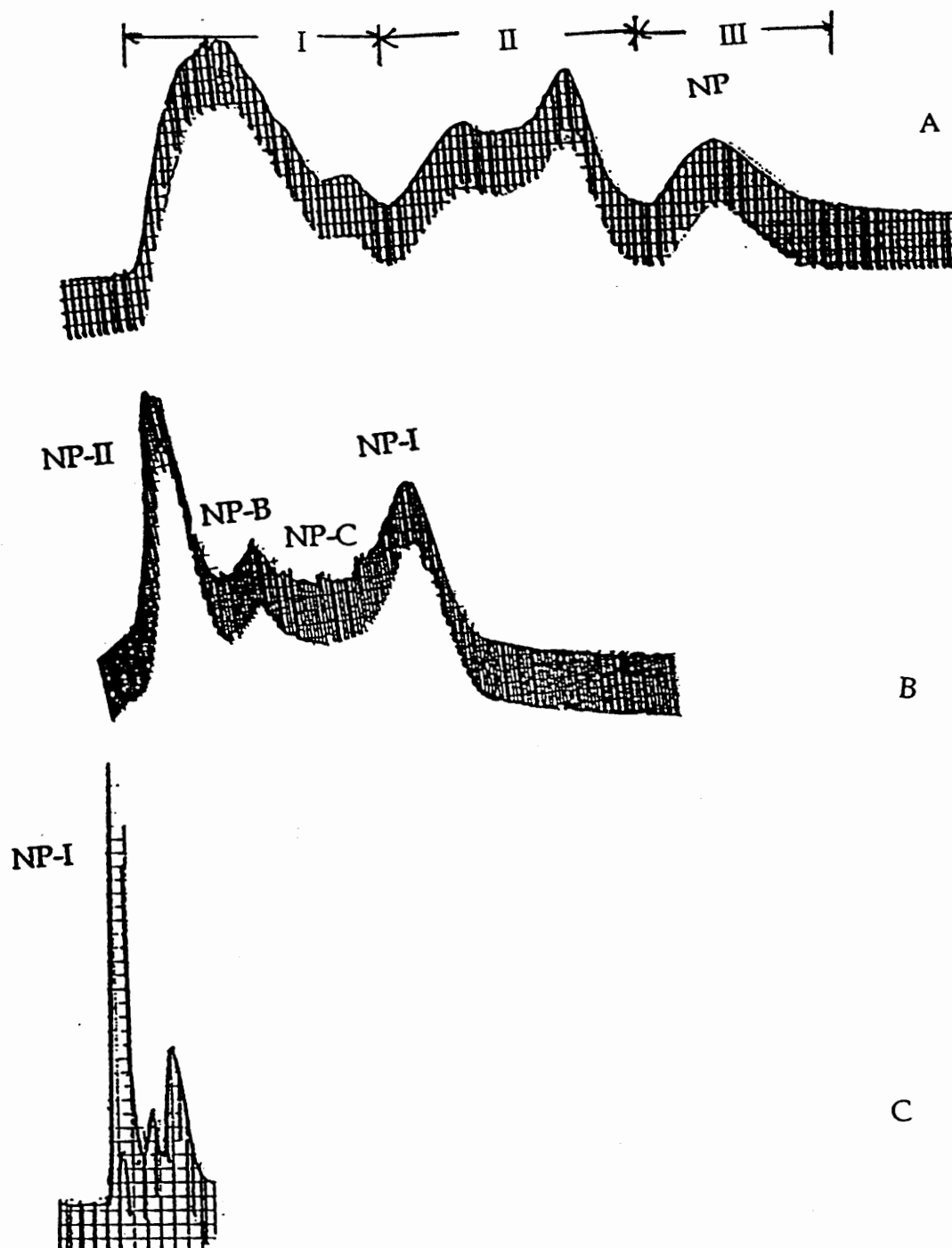


Figure 6: Column chromatography for isolation of NPs. (A) Gel filtration using Sephadex G-75. The absorbency was set at 2.0, chart speed 0.3 inch/hour, 5.5 mL / fraction. (B) Ion exchange separation using DEAE-Sephadex A-50. The absorbency was set at 0.5, chart speed 0.6 inch/hour, 5.0 mL / fraction. (C) Gel filtration using Sephadex G-25. The absorbency was set at 1.0, chart speed 0.3 inch/hour, 5.5 mL / fraction.



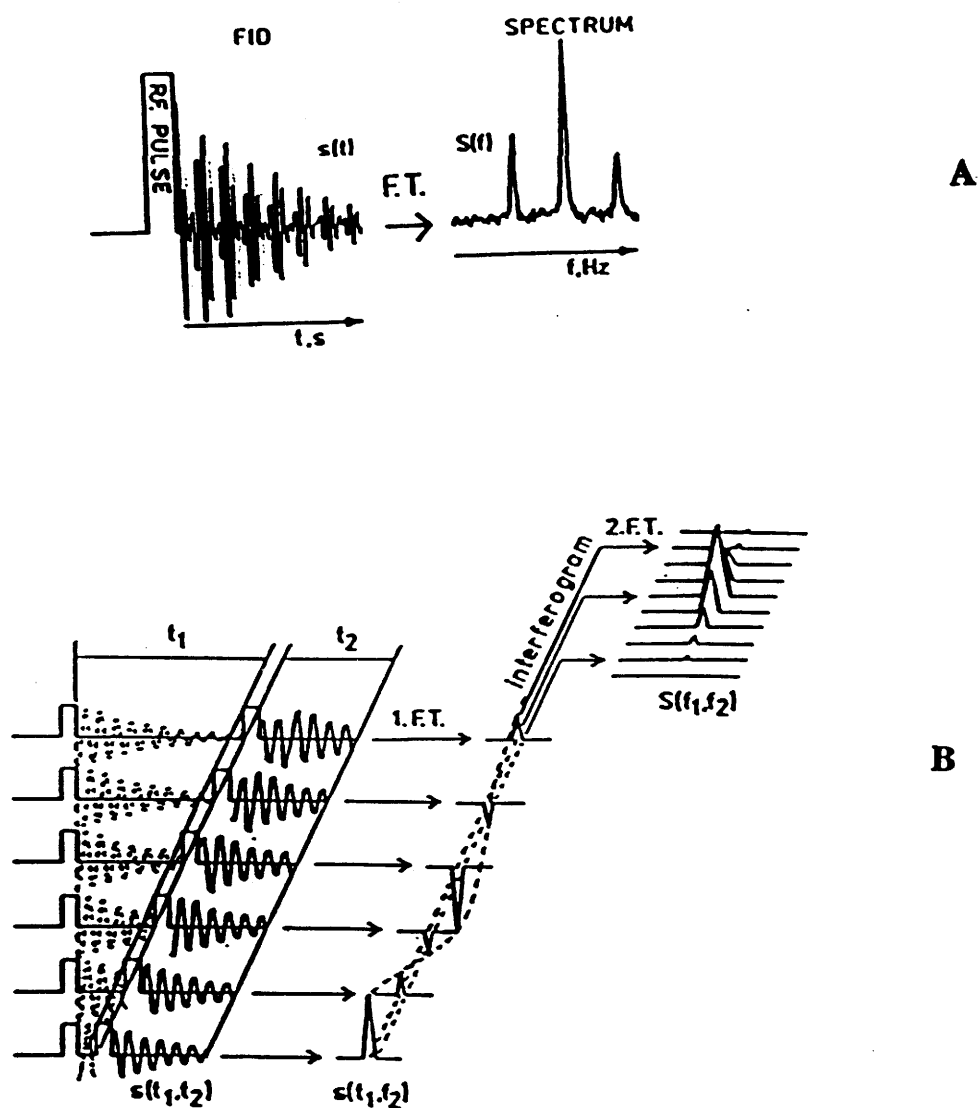


Figure 7: (A) Pulsed 1D NMR measurement. FT stands for Fourier transform. (B) Example of pulsed 2D NMR. The Figure is adapted from Bellama, 1988.

## RESULTS AND DISCUSSION

### CHAPTER 3

A set of cosolvents, each miscible with D<sub>2</sub>O, was investigated to evaluate each one's ability of lowering the NP-I dimerization constant. The <sup>1</sup>D NMR spectra of NP-I were monitored while titrating the protein with cosolvents. Since both concentration-dependent and acetonitrile-dependent spectra gave similar dimerization-sensitive NMR spectra, the perturbed resonances can be used as reference to indicate whether other cosolvents shift the dimer ⇌ monomer equilibrium toward monomer. By comparing the cosolvent-sensitive resonances to dimerization-sensitive resonances, it was concluded which cosolvent was able to shift the dimer/monomer equilibrium toward monomer. For each cosolvent, 1 mM NP-I was used (concentration is given in terms of monomer units, M<sub>r</sub> = 10,000 D). All the cosolvents were available from ISOTEK Inc., Cambridge Isotope Inc., or ALDRICH Chemical Co.. Figure 8 gives the <sup>1</sup>H NMR spectrum of each cosolvent in D<sub>2</sub>O.

#### Organic Cosolvents

##### Propionitrile

Propionitrile is homologous to acetonitrile. Figures 9, 10, and 11 show the titration results. Arrows indicate the resonances sensitive to the addition of

propionitrile. Because propionitrile has a large apolar chain than acetonitrile, it is much less miscible with D<sub>2</sub>O. When the volume of propionitrile reached 22%, two phases appeared in the solution.

As can be seen in Figure 9, there are only small chemical shift differences between spectra with and without the propionitrile cosolvent. These differences are due to the presence of the polar organic solvent. The overall preservation of the chemical shift positions under conditions with or without propionitrile indicates that the basic fold of the protein is preserved.

In proton NMR, the resonance frequencies of amide (NH) and aromatic protons are between 6 and 10 ppm. The alpha protons (H<sub>α</sub>) resonate between 4 and 6 ppm, and most of the methyl groups between 0 and 2 ppm. Subtle deviations from expected chemical shifts have been recognized as a sensitive measure of molecular conformation and environment. H<sub>α</sub>s and NHs experience an upfield shift when placed in helical conformation, and a downfield shift when placed in a β-structure (Wishart et al., 1991).

Figure 10 shows the aliphatic region. Arrows indicate propionitrile-sensitive resonances. Between 0 and 1 ppm, there are well-resolved peaks at 0.56 and 0.4 ppm. The peaks shift downfield upon addition of propionitrile, and merge with a big peak. The 0.56 ppm resonance represents a methyl resonance (Sardana and Breslow, 1982). The 0.4 ppm resonance was assigned to the βHs of Ala-68 and γHs of Thr-38 (Breslow et al., 1995). These protons overlap in 1D spectra. In the random coil the βHs of Ala are at 1.39 ppm, while the γHs of Thr are at 1.2 ppm (Wüthrich, 1986). The cause of the large upfield shift of these protons in NP-I is due to the spatial proximity to aromatic ring. The three βHs of Ala-68 sit atop the Phe-22 ring, and the γHs of Thr-38 are close to the Phe-35 ring (Breslow et al., 1995). After addition of propionitrile, the 0.56

and 0.4 ppm resonances shift to low field. This may indicate that the Phe ring that caused methyl protons upfield shift to 0.56 and 0.4 ppm in the dimer are not in such close proximity to these protons in monomer. Another reason for this chemical shift difference could be due to a three-dimensional shift within the monomer interior, involving the aromatic residues (Barbar, 1992). In the 1.1-1.4 ppm region, there are two indicators of dissociation of dimer. At about 1.21 ppm, there is an unresolved shoulder in 1 mM NP-I, which means that most of protein molecules are dimer (Peyton et al., 1986). With increasing concentration of propionitrile, this resonance is resolved and gets sharper. The resonance at 1.27 ppm in the dimer spectrum is an unresolved signal peak, but upon the addition of propionitrile, this splits into a multiple. The resonances in 1.1-1.4 ppm region were assigned to the -CH<sub>3</sub> protons of Ala-89, and Ala-90. The 1.27 ppm resonance was assigned to Thr-9 (Sardana and Breslow, 1984). The changes in 1.1-1.4 ppm region presumably reflect changes at Thr-9, because NP-I 89-92 residues do not participate NP's self-association or binding to hormone (Peyton et al., 1986). The perturbation at Thr-9 does not reflect the direct change at the interface, but reflects a dimerization-induced conformational change, because Thr-9 is distant from the monomer interface in the crystal structure (Chen et al., 1991). However, this conclusion is not strictly established (Breslow et al., 1992). Under dimer conditions, there is a resolved resonance at 1.69 ppm. When the concentration of propionitrile increases, this resonance shifts upfield and merges with a big peak. At the region around 2.4 ppm, there is resonance position that increases in intensity with increasing concentration of propionitrile. As the spectrum of propionitrile-d<sub>5</sub> (Figure 8) shows, neither of these sensitive resonances is from chemical shift interference of cosolvent itself.

Bovine NP-I contains three phenylalanine residues per chain. Since one of the Phe residues (Phe-91) can be excised without any effect on function (Rabbani, et al., 1982, Sardana and Breslow, 1984), the Phe-22 and Phe-35 residues were able to be assigned (Breslow, 1995). According to the crystal structure (Chen et al., 1991), Phe-35 is at the monomer-monomer interface. As shown in Figure 11 (aromatic region), the indication of dimer dissociation is accompanied by a marked change in Phe-35 proton signals, the 7.56 ppm peak shifts upfield, merging with large resonances. The resonance at 7.2 ppm which was assigned to Phe (sum of Phe-22 and Phe-35) gets sharper upon increasing concentration of propionitrile. The cause of these changes is due to the different environment of Phe ring protons between dimer and monomer.

There is only one tyrosine (Tyr-49) residue in bovine NP-I. This Tyr has signals of the 3,5 ring protons at 6.75 ppm and 2,6 ring protons at 7.07 ppm which are sharpened with increasing concentration of propionitrile. Under dimer conditions, there are multiple peaks at 6.75 ppm, while under monomer conditions, multiple peaks disappear accompanied by a small downfield shift. At 16% propionitrile, there is a single main resonance for the 3,5 ring protons, located at 6.83 ppm. This single peak is assigned to monomer. The multiple 3,5 ring proton peaks at 6.75 ppm were assigned to dimer (Breslow et al., 1992). The presence of resolved coexistent monomer and dimer peaks at intermediate cosolvent concentrations is in agreement with the slow rate of monomer-dimer equilibrium on the NMR time scale (Pearlmutter, 1979). The presence of multiple upfield shifted peaks in the dimer may indicate that dimerization induced two distinct slowly exchanging environmental states for 3,5 ring protons of Try-49 without significantly increasing dipolar broadening relative to monomer (Breslow et al., 1992). In the monomer, a single

conformation or, more likely, a rapid equilibrium between multiple forms is observed.

Also seen in Figure 11 are dissociation-sensitive peaks at 6.41 and 6.15 ppm. The 6.41 ppm peak decreases with increasing concentration of propionitrile cosolvent, while the 6.15 ppm peak increases intensity and shifts upfield with increased propionitrile concentration. The 6.41 ppm peak was assigned to a  $H_{\alpha}$  of dimer (Breslow et al., 1992). In the spectra recorded in the presence of intermediate concentrations of propionitrile, the coexistence of the monomer and dimer peaks reflects the slowly exchanging dimer and monomer states of the same  $H_{\alpha}$ . The ratio of these two peaks' intensities reflects the degree of dissociation of dimer quantitatively (e.g. see Nicolas et al., 1980, and Peyton et al., 1986). The degree of dissociation, as evidenced by the decrease in intensity of the 6.41 ppm peak, also correlates with the sharp Tyr 3,5 ring proton signal assigned to monomer.

Consistent with the observations in acetonitrile-dependent and concentration-dependent spectra, His-80  $C_4H$  at 7.25 ppm (overlapping with Phe protons) and  $C_2H$  at 8.5 ppm get sharper with increasing concentration of propionitrile.

Although decreasing the concentration of NP-I shifts the dimer  $\rightleftharpoons$  monomer toward monomer, the dilution of NP-I during the titration with cosolvents was not the main reason cause the dissociation-sensitive resonances shift. Because the dilution factor was not significant enough. For example, in the case of 16% propionitrile, the NP-I concentration was 0.86 mM. The dilution itself could not significantly effect the dissociation-sensitive resonances.

In summary, the spectrum of 1 mM NP-I gradually changes from mostly NP-I dimer to mostly NP-I monomer with the increasing concentration of

propionitrile. The changes in spectra upon addition of propionitrile are due to dissociation of dimer, because the spectral changes from dimer to monomer are consistent with that of concentration-dependent and acetonitrile-dependent spectra. As a cosolvent, propionitrile has the same effect as acetonitrile, which shifts the NP dimer  $\rightleftharpoons$  monomer equilibrium toward monomer. The dissociation-sensitive shifts can be observed from spectra in which both dimer and monomer resonances coexist. This also indicates that the exchange between dimer and monomer is slow enough so that the resonances coexist, resolved on the NMR time scale.

The monomer-monomer interface of the NP-I dimer is hydrophobic (Nicolas et al., 1978). Propionitrile may penetrate the hydrophobic interface, causing dissociation of dimer. As seen in the crystal structure of NP-II (Figure 1), the monomer-monomer interface is mainly comprised of  $\beta$ -sheet interactions that stabilize the dimer by hydrogen bonding (Breslow and Burman, 1990). When solvent system interacts with interface, D<sub>2</sub>O and CD<sub>3</sub>CD<sub>2</sub>CN could compete with  $\beta$ -sheet hydrogen bonds, form hydrogen bonds between solvent and monomer interface residues.

By comparing acetonitrile-titrated NP-I spectra (Figure 5) to propionitrile-titrated spectra (Figure 9), it is apparent that propionitrile dissociates the NP-I dimer at a slightly lower concentration than does acetonitrile. The degree of dissociation is compared by the relative intensity of monomer peak at 6 ppm to dimer peak at 6.4 ppm. However, pure monomer cannot be obtained by adding propionitrile because it is no longer miscible with D<sub>2</sub>O when the organic cosolvent exceeds 22%. So for the purpose of dissociating NP-I at high protein concentration, using acetonitrile remains better.

### Methanol-d<sub>4</sub>

Deuterated methanol was tried as cosolvent to break the dimerization of NP-I. Methanol has a dipole moment of 1.69, smaller than that of acetonitrile, which has a dipole moment of 4.0. Also, methanol has smaller dielectric constant of 33, compared to acetonitrile of 38 (Bruice, 1995).

1 mM NP-I was titrated with methanol at pH 6.0. As shown in Figure 12, the dissociation-sensitive resonances in the aliphatic and aromatic regions were examined. In the region between 0 and 1 ppm, the resonance at around 0.55 ppm shifts downfield and splits. The downfield shifts for Ala-68 and Thr-38 resonances at around 0.4 ppm are not as large shift as in acetonitrile or propionitrile cosolvent. The increasing sharp peak at about 1.2 ppm is observed in the intermediate concentration of methanol (8.5%), but this disappears at high methanol concentration (16%). Similar to the observation in nitrile cosolvents, the resonance at 1.73 ppm is well resolved in low methanol concentration, then loses resolution with increasing concentration of methanol. Unlike nitrile cosolvents, the resonance around 2.43 ppm lost resolution at high concentration of methanol. The Tyr-49 3,5 and 2,6 ring protons at 6.72 and 7.1 ppm get sharper upon increasing concentration of methanol. Also the aromatic region between 6.9 and 7.5 ppm broadens and loses resolution. This might be due to the partial denaturation of protein. The dimer peak at 6.40 ppm remains almost the same intensity, indicating that the dimerization constant did not change significantly upon the addition of methanol.

Methanol has a similar dielectric constant to nitriles and it is structurally similar to nitriles (Table I). Because of the similarity, methanol could have the



ability to dissociate the dimer (a few dissociation-sensitive resonances were observed). Yet protein seems denatured at relatively low methanol concentration compared to nitriles. Probably, because methanol is a stronger hydrogen-bond former than nitriles, it penetrates into the interior of protein, solvating the hydrophobic interior, and severely disturbs its structure.

### Dimethyl Sulfoxide -d<sub>6</sub>

DMSO has some similar physical properties to acetonitrile. Both have high dipole moment, DMSO 3.9, and acetonitrile 4.0. Both have a similar dielectric constant, DMSO 46, and acetonitrile 38 (Bruice, 1995). Yet their structures are totally different.

Spectra of 1 mM NP-I were recorded with increasing volume percentage of DMSO. As shown in Figure 13, all the dissociation-sensitive resonances remain unchanged with increasing concentration of DMSO. The distortion in peak shape (See Appendix) might be due to denaturation of protein at the very high volume percent concentration (43%) of DMSO. After the volume percentage of DMSO exceeded 43%, two phases appeared in the NMR tube. Thus DMSO fails to shift the NP-I dimer  $\rightleftharpoons$  monomer toward monomer, likely because DMSO might not bind to the monomer-monomer interface of dimer.

### Ethyl Acetate -d<sub>8</sub>

Ethyl acetate has much lower dielectric constant, which is 6, than acetonitrile. Ethyl acetate is also structurally different from acetonitrile.

Figure 14 shows the titration of 1 mM NP-I with increasing volume percentage of ethyl acetate. No obvious and large changes in dissociate-

sensitive resonance are observed. When the concentration of ethyl acetate exceeded 16%, it was no longer miscible with D<sub>2</sub>O, two phases separated in the NMR tube. Thus, ethyl acetate cannot break the NP dimer.

### Cosolvent effects

Among the cosolvents, CD<sub>3</sub>CN, CD<sub>3</sub>CD<sub>2</sub>CN, CD<sub>3</sub>OD, CD<sub>3</sub>SOCD<sub>3</sub>, and CD<sub>3</sub>COOCD<sub>2</sub>CD<sub>3</sub>, used in this work, it was found that only CD<sub>3</sub>CN and CD<sub>3</sub>CD<sub>2</sub>CN can significantly shift the NP-I dimer  $\rightleftharpoons$  monomer equilibrium toward monomer. This might be due to physical properties and structural feature of nitriles. Table I lists some physical properties of each cosolvent and their ability to dissociate NP-I dimer. The ability of dissociation of NP-I dimer is estimated from the ratio of intensity of Tyr-49 3,6 ring protons at 6.8 ppm to the intensity of dimer peak at 6.4 ppm. The numbers indicate approximately how much volume percentage cosolvent is needed to low the intensity ratio to half. Figure 15 shows the relation between dielectric constant, dipole moment of cosolvent and its ability to dissociate the dimer. In the Figure, 'NO' in "Ability" indicates that cosolvent cannot dissociate NP dimer at the concentration miscible to D<sub>2</sub>O.

Since the monomer-monomer interface is more hydrophobic than electrostatic (Nicolas et al., 1978), a high dielectric constant solvent like water, has little effect on the interface. Lowering the dielectric constant of the solvent system affects the strengths of hydrophobic interaction. Addition of substantial quantities of polar organic cosolvents to water decreases the bulk dielectric constant of the medium and thus disrupt the hydrophobic force. On the other hand, the  $\beta$ -sheet structure of monomer-monomer interface stabilized by hydrogen bonds. The solvent system has to have some polarity

to disturb the hydrogen bond. Thus, the monomer-monomer interface is sensitive to the dielectric constant of the solvent system which should be low to destabilize the hydrophobic force, but also should be high enough to disrupt hydrogen bond. The moderately high dielectric constant of nitriles gives the solvent system appropriate polarity to disturb the hydrogen bonding of the interface, while also destabilizing the hydrophobic interactions. Also, nitriles can form hydrogen bond with interface residues. The relative longer apolar chain of propionitrile probably makes it easier to penetrate the monomer-monomer interface than does acetonitrile. So at the same concentration, propionitrile shifts the dimer  $\rightleftharpoons$  monomer equilibrium toward monomer slightly more than acetonitrile does. But also because the longer apolar chain, propionitrile is less miscible with D<sub>2</sub>O. Thus a very high weight percentage of NP-I monomer only can be obtained in D<sub>2</sub>O/ acetonitrile solvent system.

Methanol (CD<sub>3</sub>OD) is a stronger competitive hydrogen-bond former than nitriles, and has an apolar chain similar to CD<sub>3</sub>CN. Methanol showed a tendency to dissociate dimer (a few dissociation-sensitive resonances changes were observed), but it also denatured the protein. At 16% methanol concentration, a significant loss in resolution of the aromatic region was observed. This could be due to the denaturation of protein.

DMSO has some physical properties similar to the nitriles, but is structurally different. As shown in Figure 15, cosolvents with moderately dielectric constant dissociate dimer at relatively low concentration, with the exception of DMSO. This is probably due to the structure of DMSO. Cosolvents that have a relative long apolar chain structure favor the dissociation. Cosolvents like DMSO which do not have an asymmetric chain structure, may not penetrate

the monomer-monomer interface, even it has appropriate polarity. And it likely denatures the protein at high concentration.

Ethyl acetate is different from nitriles both in physical and structure properties. Thus it could not dissociate NP-I dimer at the concentrations miscible with D<sub>2</sub>O (Figure 15).

In summary, the equilibrium between dimer and monomer is sensitive to the solvent character and structure. Only when the solvent system has appropriate polarity and the cosolvent has linear apolar chain, NP monomer-monomer interface could be penetrated by solvent, shift the dimer  $\rightleftharpoons$  monomer equilibrium toward monomer.

Table II summarizes the response of NP-I dissociation-sensitive resonances upon addition of each cosolvent, as well as the assignments of some resonances.

TABLE I  
Physical Properties of Cosolvents and Ability to Dissociate NP  
Dimer <sup>a</sup>

|                  | Dipole<br>Moment | Dielectric<br>Constant | H Bond | Breakage<br>of Dimer<br><sup>b</sup> |
|------------------|------------------|------------------------|--------|--------------------------------------|
| Acetonitrile     | 4.0 D            | 38                     | Yes    | 12%                                  |
| Propionitrile    | 3.5 D            | 28.9                   | Yes    | 11%                                  |
| Methanol         | 1.7 D            | 32.7                   | Yes    | 17%                                  |
| DMSO             | 3.9 D            | 46.3                   | Yes    | NO                                   |
| Ethyl<br>Acetate | 1.8 D            | 6                      | Yes    | NO                                   |
| Water            | 1.9 D            | 78                     | Yes    | NO                                   |

a. Data were obtained from Riddick, J. A. et al., Organic Solvents Physical Properties and Methods of Purification, (1986), and from Bruice, P. Y. Organic Chemistry, (1995).

b. Estimated percentage of cosolvent is needed to decrease the ratio of Tyr-49 3,5 ring Hs / 6.4 ppm resonance to half.

TABLE II  
List of Dimer/Monomer Sensitive Resonances

| Chem Shift                     | 0-1ppm                          | 1.1-1.4                   | 1.7                         | 2.5              | 6.1                       | 6.4                         | 6.8               | 7.1          | 7.3                          | 7.6          | 8.4                     |
|--------------------------------|---------------------------------|---------------------------|-----------------------------|------------------|---------------------------|-----------------------------|-------------------|--------------|------------------------------|--------------|-------------------------|
| Assignment                     | T38 $\beta$ H<br>A68 $\gamma$ H | T9                        | ? <sup>a</sup>              | ?                | H $\alpha$                | H $\alpha$                  | Y49<br>3,5        | Y49<br>2,6   | H80<br>C <sub>4</sub> H<br>F | F35          | H80<br>C <sub>2</sub> H |
| $\downarrow$ [NP] <sup>h</sup> | D.S. <sup>b</sup>               | $\uparrow$ R <sup>c</sup> | $\downarrow$ R <sup>d</sup> | $\uparrow$ R     | $\uparrow$ I <sup>e</sup> | $\downarrow$ I <sup>f</sup> | s.p. <sup>g</sup> | $\uparrow$ R | $\uparrow$ R                 | $\uparrow$ R | ---                     |
| Acetonitrile                   | D.S.                            | $\uparrow$ R              | $\downarrow$ R              | $\uparrow$ R     | $\uparrow$ I              | $\downarrow$ I              | s.p               | $\uparrow$ R | $\uparrow$ R                 | D.S.         | S                       |
| Propionitrile                  | D.S.                            | $\uparrow$ R              | $\downarrow$ R              | $\uparrow$ R     | $\uparrow$ I              | $\downarrow$ I              | s.p               | $\uparrow$ R | $\uparrow$ R                 | D.S.         | S                       |
| Methanol                       | D.S.                            | $\uparrow$ R              | $\downarrow$ R              | --- <sup>i</sup> | ---                       | ---                         | ---               | ---          | ---                          | ---          | ---                     |
| DMSO                           | ---                             | ---                       | ---                         | ---              | ---                       | ---                         | ---               | ---          | ---                          | ---          | ---                     |
| Ethyl acetate                  | D.S.                            | ---                       | ---                         | ---              | ---                       | ---                         | ---               | ---          | ---                          | ---          | S                       |

a. have not be assigned    b. Downfield Shift    c. Increase Resolution    d. Decrease Resolution    e. Increase Intensity    f. Decrease Intensity    g. Single Peak    h. Dcrease concentration of NP    i. no change

### Binding of Acetonitrile at the NP Monomer-Monomer Interface

From the above 1 D NMR studies, only the structurally and physically similar cosolvents, acetonitrile and propionitrile, could break the dimerization of NP-I. These cosolvents affect the NP dimer by changing the character of the solvent. A question was raised as to whether these cosolvents affect the dimer also by binding to the monomer-monomer interface. Thus a further investigation of the cosolvent effect was done by 2 D NOESY. As stated earlier (Chapter 2), NOESY provides information about protons that are close in space, as well as about chemical exchanges between protons. Because acetonitrile and propionitrile have a similar function in dissociating the dimer, and because acetonitrile is more miscible with water, acetonitrile was used as the cosolvent in the following 2 D NOESY studies.

At an intermediate concentration (10% volume percentage) of acetonitrile, and 1 mM total NP-I, NP-I dimer and monomer coexist. 2 D NOESY spectra for 1 mM NP-I, with and without the 10% acetonitrile- $d_3$  cosolvent, were performed. Figure 16 and 17 show the comparison between NP-I without acetonitrile- $d_3$  and with acetonitrile- $d_3$ . In general, cross-peaks in NP-I with acetonitrile- $d_3$  are sharper and more resolved. Some peaks are downfield shift due to the presence of acetonitrile. For example, the cross-peak at 6.7/7.1 ppm in Figure 16 gets sharper and shifts downfield to 6.8/7.2 ppm in Figure 17.

To observe specific bonding of acetonitrile to protein, 2 D NOESY spectra of NP-I with 10%  $CH_3CN$  was performed. If acetonitrile has specific bonding site(s) at monomer-monomer interface, the cross-peaks between certain interface-residues of NP-I and  $CH_3CN$  would be observed because of proximity in space, specifically, if the  $CH_3CN$  is bound with a sufficiently long residence time. Figure 17 and 18 show 2 D NOESY

spectra of NP-I with 10% CD<sub>3</sub>CN and 10% CH<sub>3</sub>CN, receptively. Figure 17A and 18A are corresponding cross sections parallel to f<sub>2</sub> axis taken at the frequency of acetonitrile at 1.95 ppm. By comparing these two spectra, it is observed that there is an extra peak in Figure 18A at about 0.6 ppm. This resonance is the sum of  $\gamma$ Hs of Ala-68 and  $\beta$ Hs of Thr-38. So at least one of these protons is close in space to CH<sub>3</sub>CN. Because Thr-38 is the monomer-monomer interface residue, it is possible that CH<sub>3</sub>CN bonds to Thr-38. From this observation, it may be concluded that the way acetonitrile dissociates NP-I is by penetrating monomer interface and binding to the interface residues.



## Denaturing Reagents

Guanidine-HCl (Gnd·HCl) and urea are denaturing reagents (also called hydrogen-bond-breaking agents). They disrupt only secondary and tertiary structure, but not primary structure, because they do not cleave covalent bonds. Urea and Gnd·HCl are both competitive hydrogen-bonding formers and hydrophobic effectors [increase the solubility of a hydrocarbon-like model compound over the solubility of this compound in pure water alone (Hippel et al., 1969)]. High concentrations of urea or Gnd·HCl allow water molecules to penetrate into the interior of proteins and solvate nonpolar side chains, thereby disrupting the hydrophobic interactions that stabilize the native conformation (Horton 1994). Gnd·HCl is also a salt. It exhibits striking specific effect on the conformation of proteins. This effect seems to have nothing to do with the sign or magnitude of protein net charge. (Hippel et al., 1969).

### Guanidine·DCl

Figure 19 shows the spectrum of Gnd·DCl in D<sub>2</sub>O. The big peak between 7 and 6 ppm is from residual Gnd·HCl, and interferes the observation of dissociation-sensitive resonance of NP-I in this region.

Figure 20 shows the titration of NP-I with increasing amounts of Gnd·DCl, from 0 M to 7 M. In the upfield region, the resonances at 0.64 ppm in the spectrum of NP-I without Gnd·DCl split and shift downfield with increasing concentration of Gnd·DCl. A similar effect is seen for the H<sub>γ</sub> of Thr-38 and H<sub>β</sub> of Ala-38 at 0.5 ppm. Also the resonance at 1.2 ppm gets sharper. When the concentration of Gnd·DCl reaches 5 M, the split resonances at 0.7 ppm become

primarily one sharp peak. The resonance at around 1.8 ppm is resolved at 0 M Gnd·DCl as in all of the dimer spectra. With increasing concentration of Gnd·DCl, this resonance merges with an upfield peak. This is similar to the changes that happen in acetonitrile and propionitrile cosolvent spectra. In the region between 2.5 and 3.0 ppm, the resonances become more resolved with increasing concentration of Gnd·DCl, also as in nitrile cosolvents. Therefore, the in aliphatic region, dissociation-sensitive resonances are sensitive to the presence of Gnd·DCl. In the aromatic region, because of the interference of Gnd·DCl chemical shifts, it is hard to analyze dissociation-sensitive changes. Yet it still can be observed that the shapes of peaks are distorted in the presence of high concentration Gnd·DCl.

By comparing 2 D NOESY spectra of NP-I, with and without 7 M Gnd·DCl (Figure 21), it can be seen that the spectrum with 7 M Gnd·DCl has many fewer cross-peaks than that of NP-I without Gnd·DCl. This probably indicates a lack of structural detail, which means protein is partly unfolded in the high concentration of Gnd·DCl. Figure 22 shows the 1 D reference and 2 D ROESY spectra of NP-I in 2.5 M Gnd·DCl. As introduced earlier, ROESY spectrum gives information about chemical exchange. This experiment was run at 45 °C, which is higher than the other spectra in this work. At the elevated temperature, the chemical exchange increases, giving more exchange cross-peaks. Also, a saturation-transfer technique was used in the spectrum. Saturation water resonance at 4.75 ppm caused saturation transfer to big peaks at 6.5 to 6.8 ppm from Gnd·DCl. Thus interference from Gnd·DCl is minimized. From Figure 22A, the dimer peak at 6.38 ppm much larger than the monomer peak at 6.1 ppm. This means that NP-I is mostly dimer in 2.5 M Gnd·DCl and 45 °C at pH 6. At this Gnd·DCl concentration, there is also an

equilibrium between folded and unfolded NP-I, as evidenced by the exchange cross-peak at 0.5/1.35 ppm. The resonance at 0.5 ppm is sum of the  $H_\gamma$  of Thr-38 and  $H_\beta$  of Ala-68 (Breslow et al., 1995). Upon addition of Gnd·DCl, the protein begins to unfold, causing the exchange of at least one of these protons between two different environments, one in the native protein environment, the other in the unfolded protein environment. Thr-38 is at the monomer-monomer interface (Breslow et al., 1995). Another exchange cross-peak is observed at 6.83/7.50 ppm. The Phe-35 ring protons thus exchange between native and unfolding environment. Phe-35 is located at the monomer-monomer interface according to the crystal structure (Chen et al., 1991). These observations indicate that unfolding of protein involves the monomer-monomer interface. Upon partly unfolding the interface, Gnd·DCl likely dissociates the dimer. The dissociation of dimer is evidenced by the changes in dissociation-sensitive resonances which are similar to those in the acetonitrile and propionitrile cosolvent. The large magnitude of spectral changes and peak distortion upon addition of Gnd·DCl is severe enough to make it difficult to state with certainty that the protein is monomer (however unlikely a dimer would be under these conditions). On the other hand, there is no evidence that dissociation of the dimer occurs before partial protein unfolding.

### Urea

Urea is also a reagent which disrupts noncovalent interaction in proteins. The spectrum (Figure 23) of urea at 25 °C and pH 6.0 shows the region between 6.3 and 4 ppm will interfere with the observation of dissociation-sensitive resonances.

Figure 24 shows a set of 1 D spectra of NP-I titrated with an increasing molar concentration of urea at 25 °C and pH 6.0. Similar changes in the aliphatic region are observed to the changes observed above for Gnd·DCl. Because of the urea interference, changes in aromatic region are hard to observe. The distorted peak shape in high concentration of urea is probably due to the unfolding of protein. Urea has a similar function as does Gnd·DCl. Both dissociate dimer simultaneously with unfolding NP-I. Gnd·DCl is more efficient, because it not only has the capacity of hydrogen-bonding and hydrophobic affector but is also a salt.

## Dye

### Bromophenol blue

Interaction of the bromophenol blue with NP was first suggested by the fact that the degree of electrophoretic separation of different rat neurophysins was dependent on concentration of bromophenol blue as a tracking dye (Burtford and Pikering, 1972). One study showed that dye was bound predominantly to the dimeric form of the protein, based on ultracentrifugation studies (Carlson et al., 1981). However, another study suggested that dye thermodynamically prefers monomer, based on spectroscopically derived binding isotherms (Pearlmutter, 1979). I attempted to use NMR in this study to understand the effects of bromophenol blue on NP. Titration of 2.7 mM NP-I with an increasing concentration of dye were carried out from 1:0.4 to 1:4 molar ratio at 25 °C and pH 2.3. At pH 2.3, NP-I has the highest affinity for bromophenol blue (Carlson et al., 1981). Figure 25 shows the spectrum of 1 mM bromophenol blue at pH 2.3. Note the dye peaks around aromatic region. Even at very low concentration of dye (NP:dye = 1:0.4), the NP-dye complex started to precipitate. As shown in Figure 26, the spectra become distorted with the increasing concentration of dye. When the NP-to-dye molar ratio reached 1:4, little or no NMR signal for the NP-dye complex existed in solution, as shown in the spectra. After the pH was raised to 7, the precipitated protein was soluble again, but dye-binding was greatly diminished. This is evidenced by the easy remove of the dye by dialysis. Consequently the dye-NP complex was not studied further. Figure 27A shows the spectrum of 0.2 mM NP-I dialyzed from pH 7.0 phosphate buffer to remove the dye. It was clean NP-I.

In order to study the NP-dye complex by NMR, further effort was made by lower the concentration of NP-I to 0.2 mM. The NP-dye was still insoluble when 0.2 mM dye was added. As shown in Figure 27B, the spectral intensity decreased by 30% when NP-dye molar ratio is 1:1. The intensity kept decreasing with increasing the concentration of dye.

So at the NP concentrations studied by solution-state NMR, the NP-dye complex was not soluble. The concentrations used in previous NP-dye studies were on the order of  $10^{-2}$  mM (e.g. see Carlson et al., 1981 and Pearlman et al., 1979). Nevertheless, our results may call for a re-evaluation of those studies and their validity.

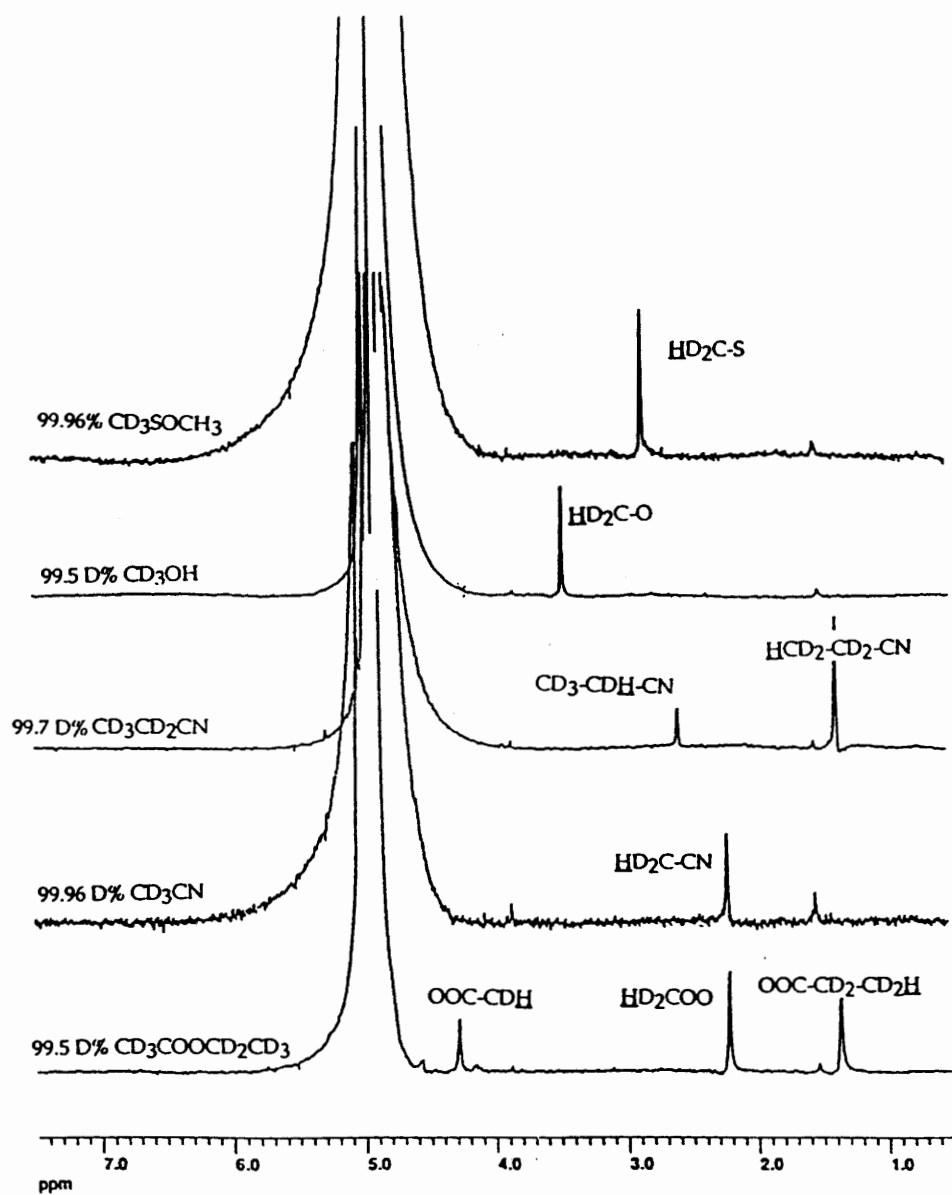


Figure 8: Spectrum of deuterated cosolvents in D<sub>2</sub>O.

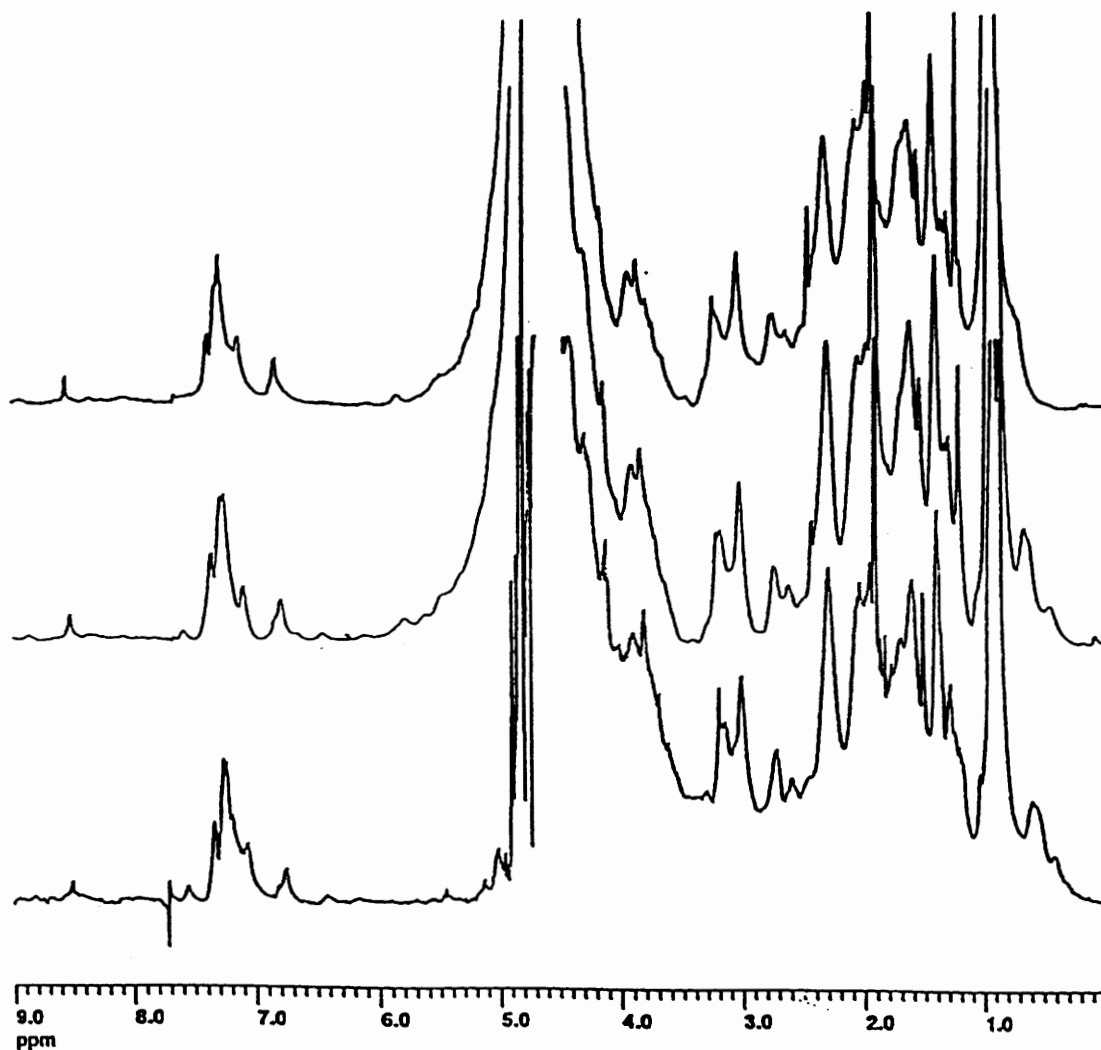


Figure 9: <sup>1</sup>H NMR spectra of propionitrile titration of 1 mM NP-I in D<sub>2</sub>O. Spectra were obtained at 25 °C, pH 6.0 in increasing concentration of propionitrile from 0% (bottom), 8.5% to 16% (top).



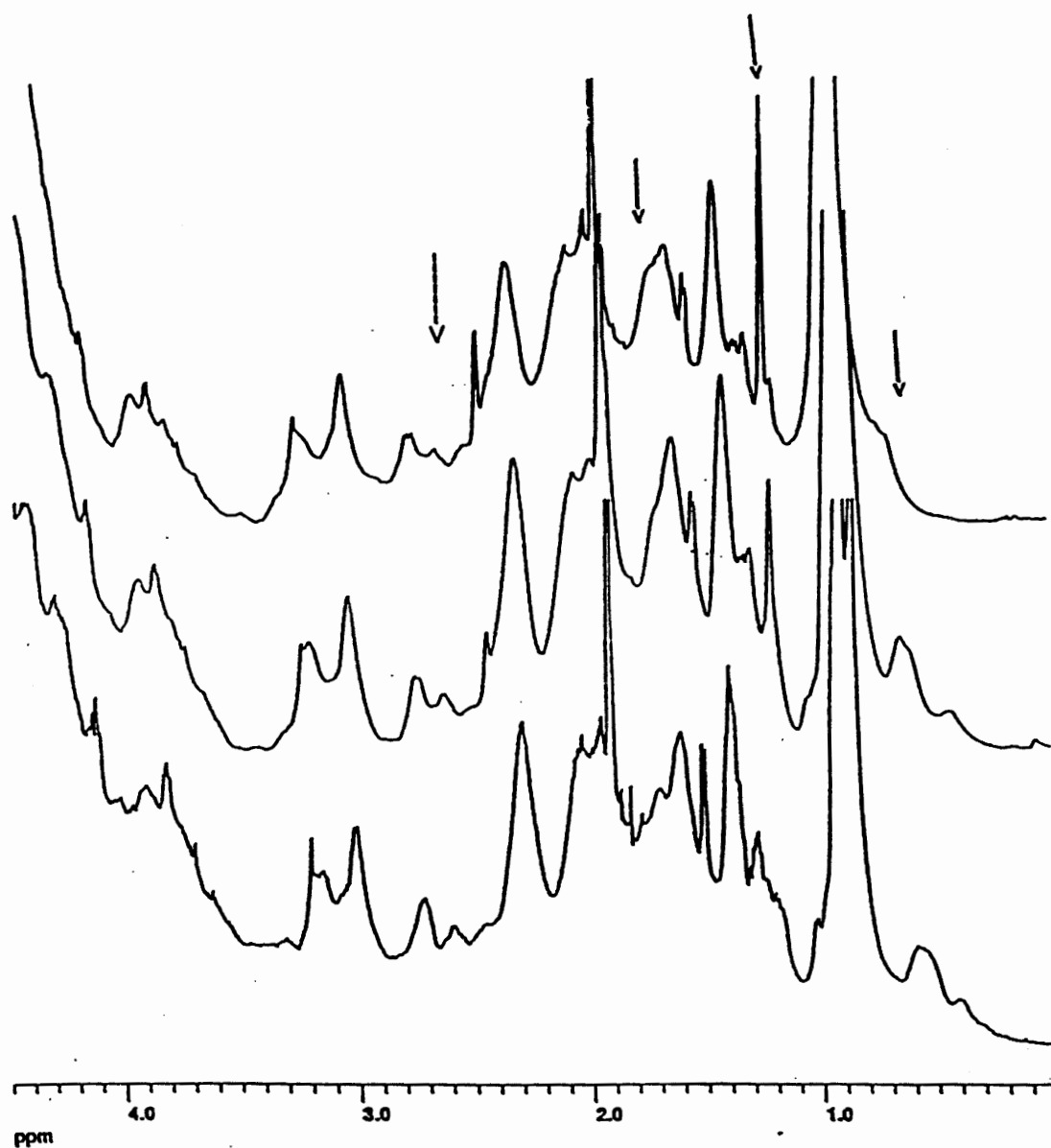


Figure 10: Aliphatic region spectra of propionitrile titration of 1 mM NP-1 in  $\text{D}_2\text{O}$ . Arrows indicates sensitive resonances. Concentration of propionitrile from bottom to top: 0%, 8.5%, and 16%.

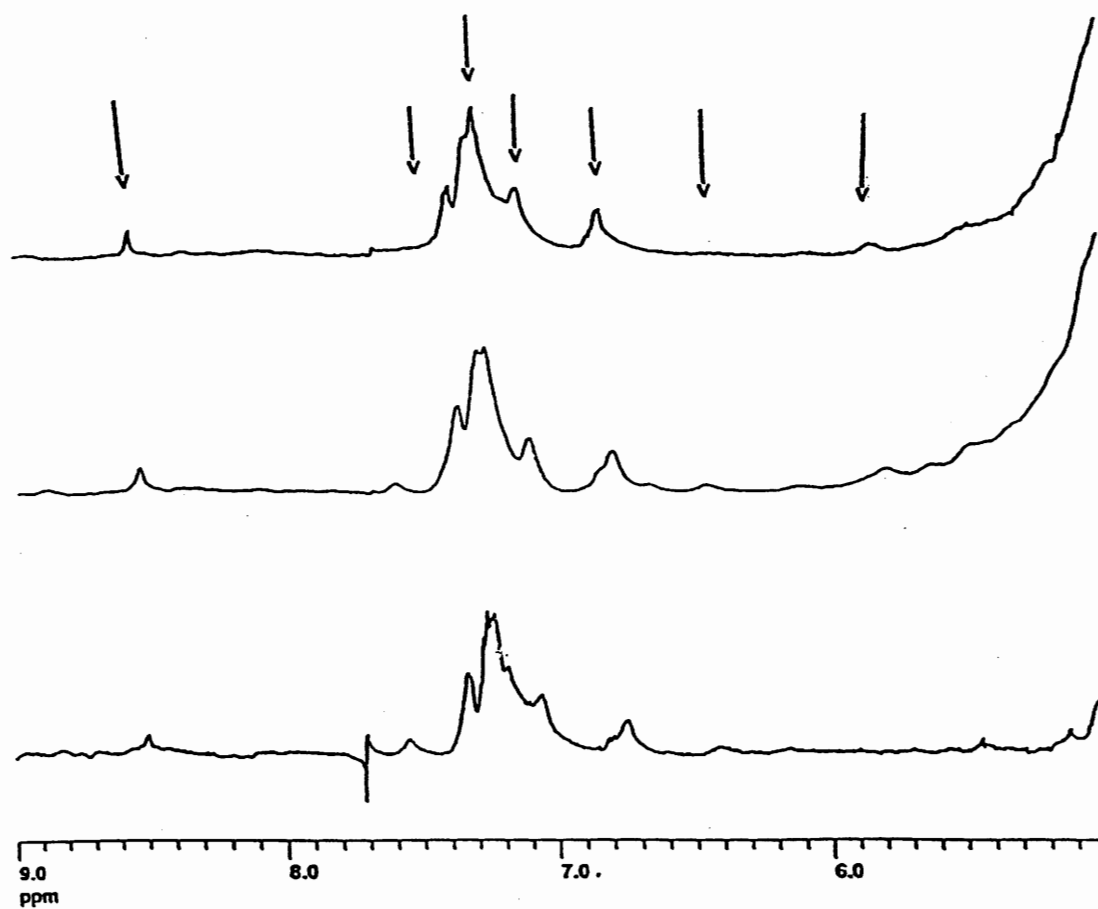


Figure 11: Aromatic region spectra of propionitrile titration of 1 mM NP-I in  $\text{D}_2\text{O}$ . Concentration of propionitrile from bottom to top: 0%, 8.5%, and 16%.

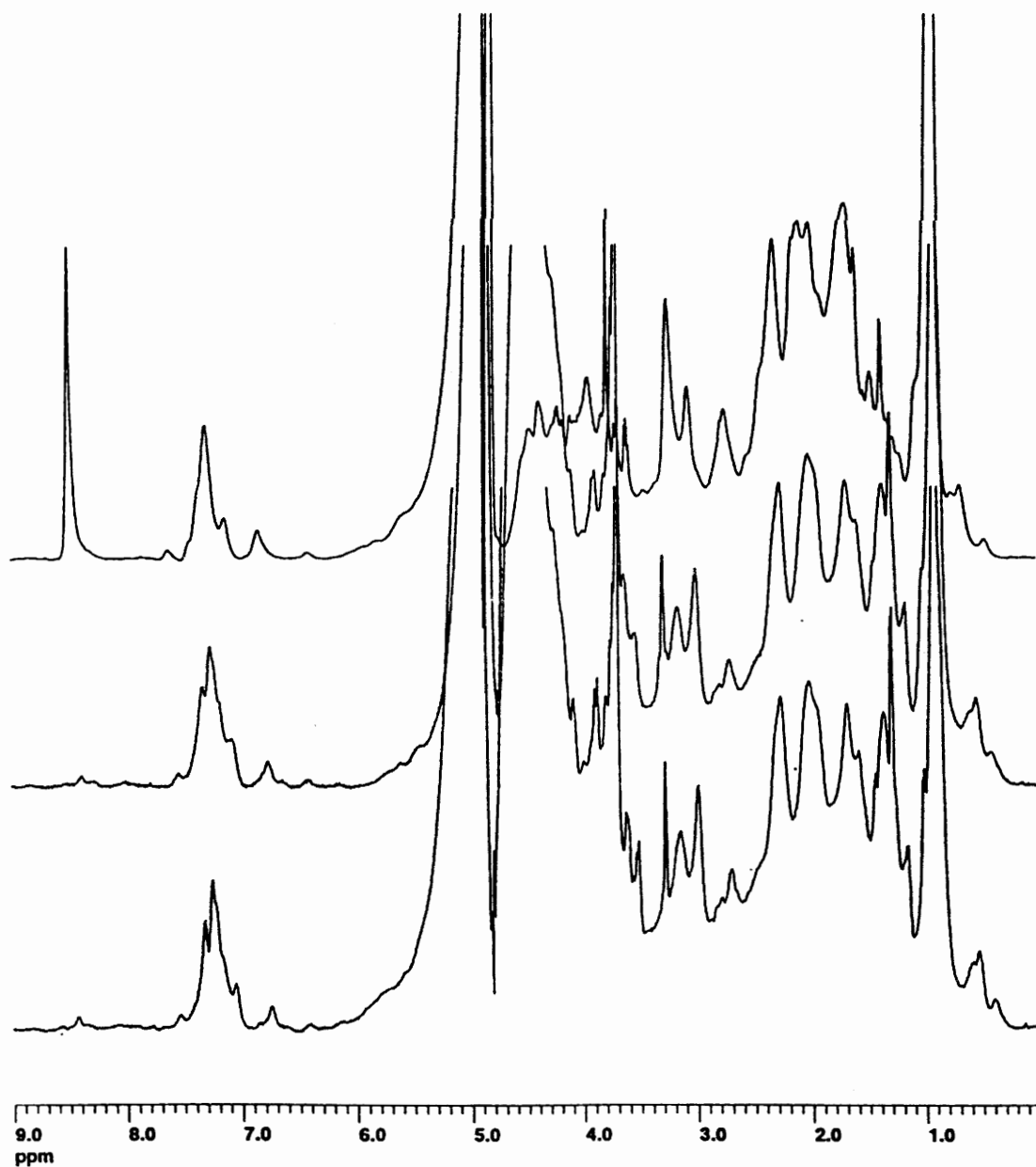


Figure 12: 1D spectra showing methanol titration of NP-I in D<sub>2</sub>O. Spectra were obtained at 25 °C and pH 6.0 from 0% methanol (bottom), 8.5% to 16% (top).

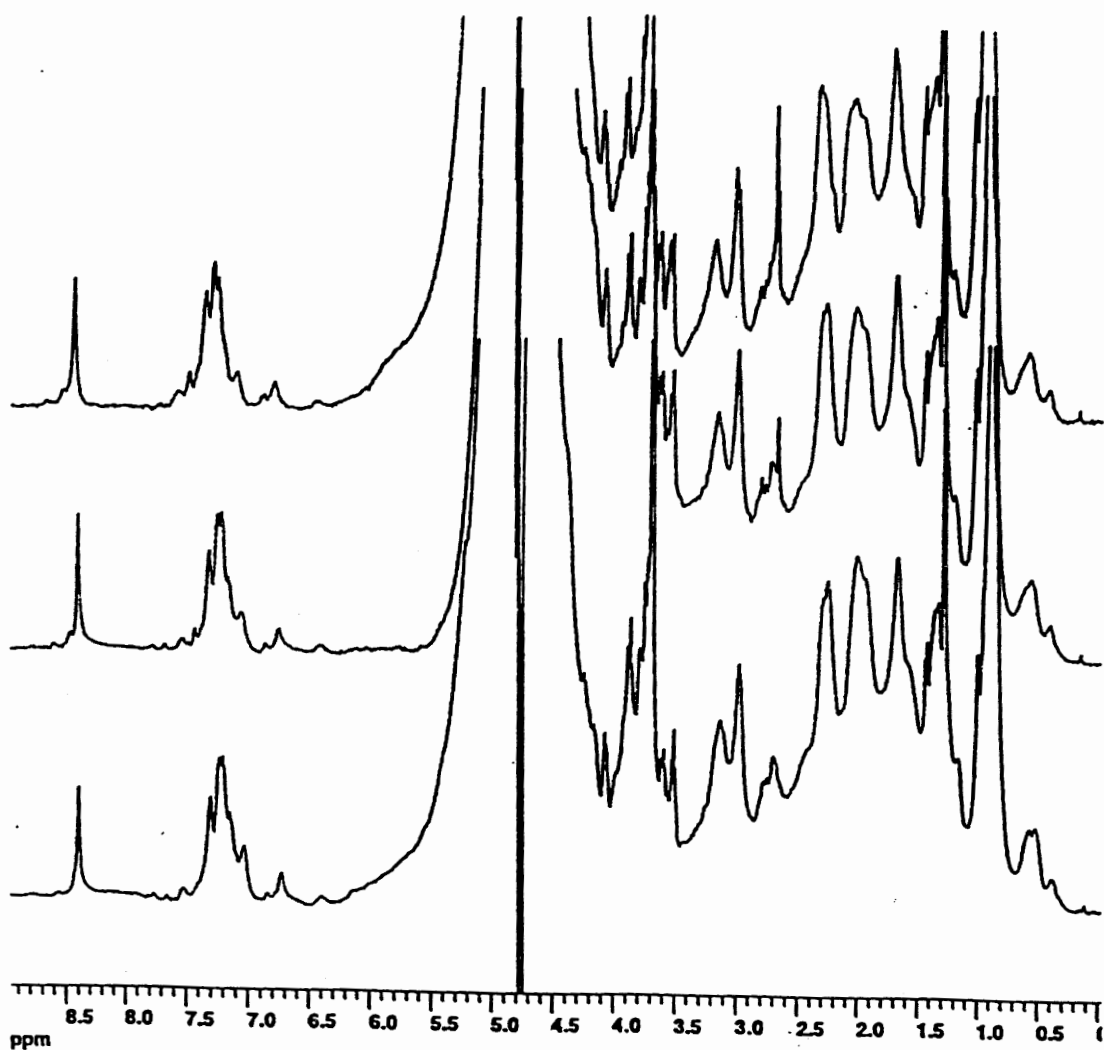


Figure 13: 1D spectra showing dimethyl sulfoxide titration of NP-I in D<sub>2</sub>O. Spectra were obtained at 25 °C and pH 6.0 from 0% DMSO (bottom), 6%, to 17% (top).

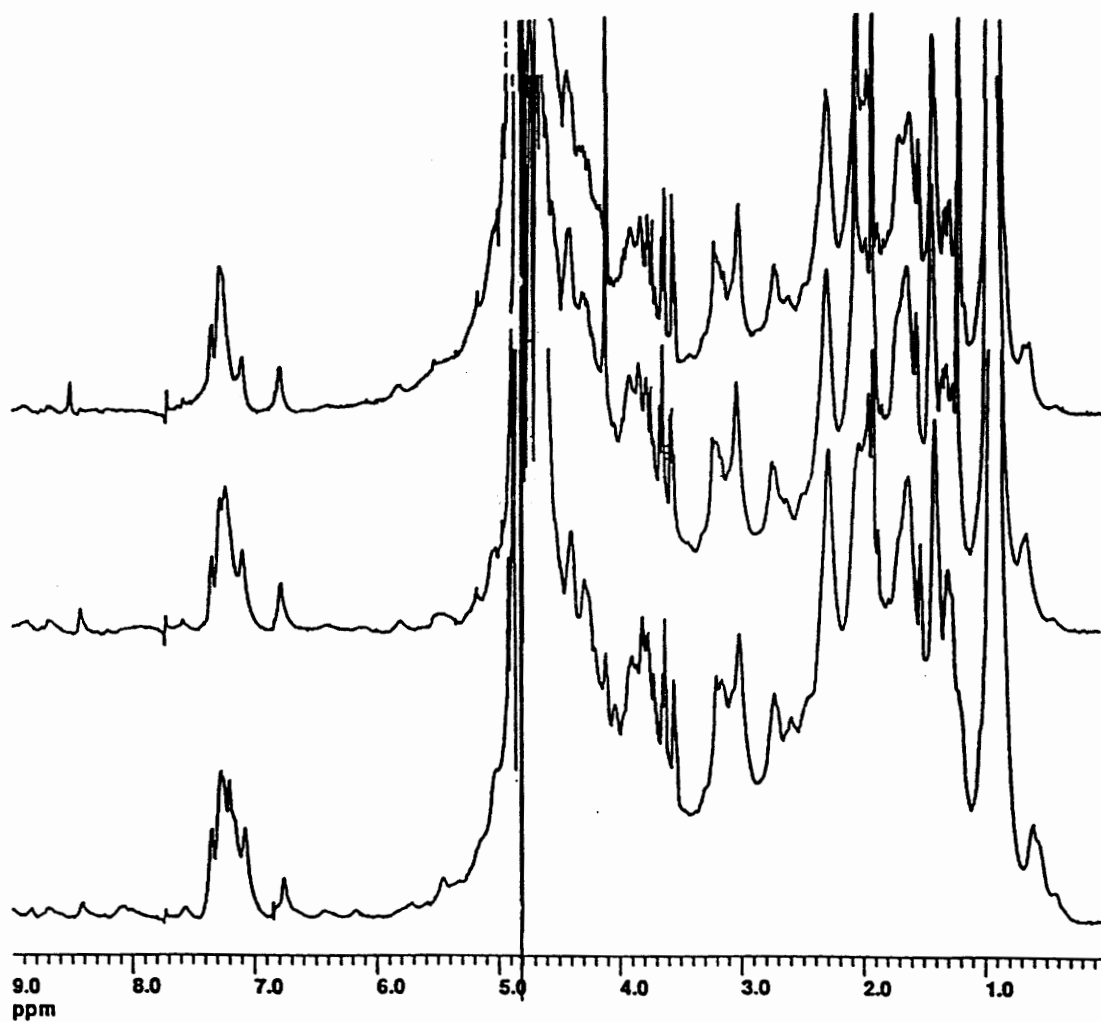


Figure 14: 1D spectra showing ethyl acetate titration of NP-I in D<sub>2</sub>O. Spectra were obtained at 25 °C and pH 6.0 from 0% ethyl acetate (bottom), 8.5% to 16% (top).

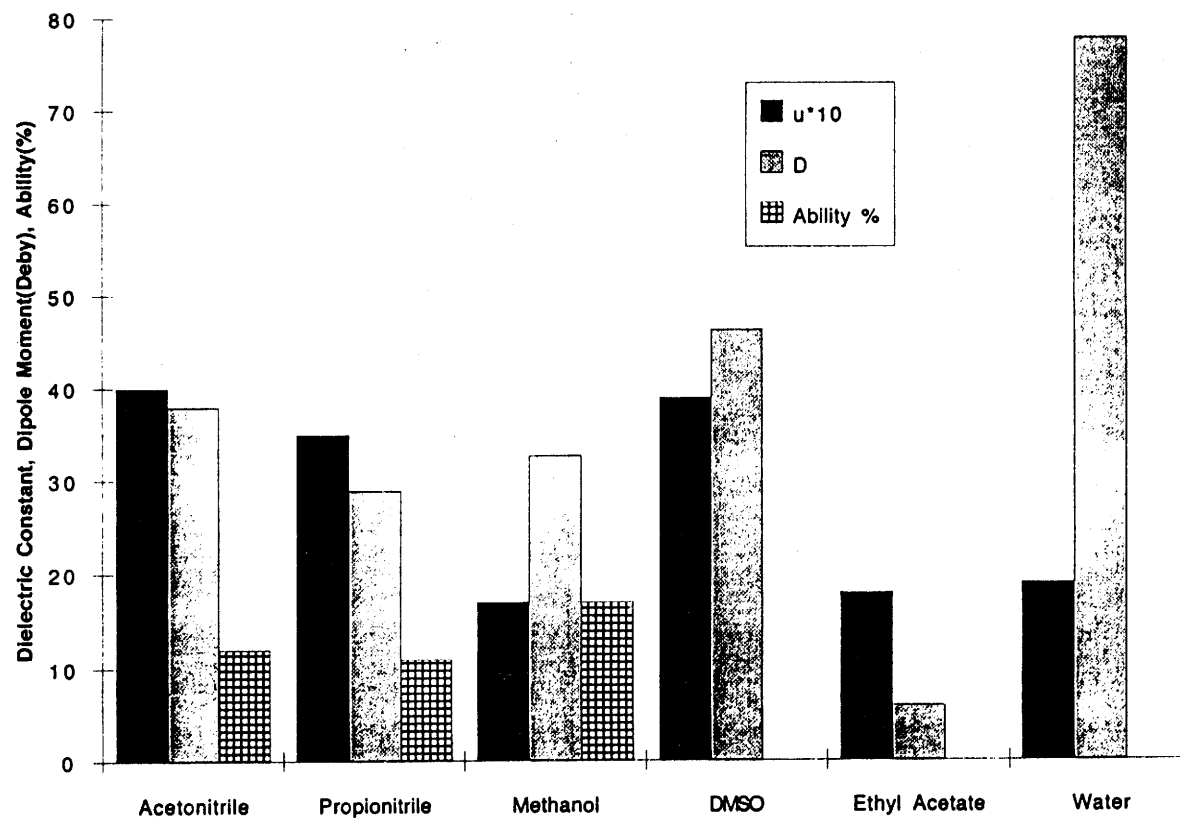


Figure 15: Relation between physical properties of cosolvent and its ability to dissociate dimer. "D" refers to dielectric constant. "u" refers to dipole moment. "Ability" indicates how much cosolvent is needed to decrease the intensity ratio of Tyr-49 3,6 ring Hs/dimer peak at 6.4 ppm to half. "NO" in "Ability" indicate that the solvent can not break the NP dimer at concentration miscible with D<sub>2</sub>O.

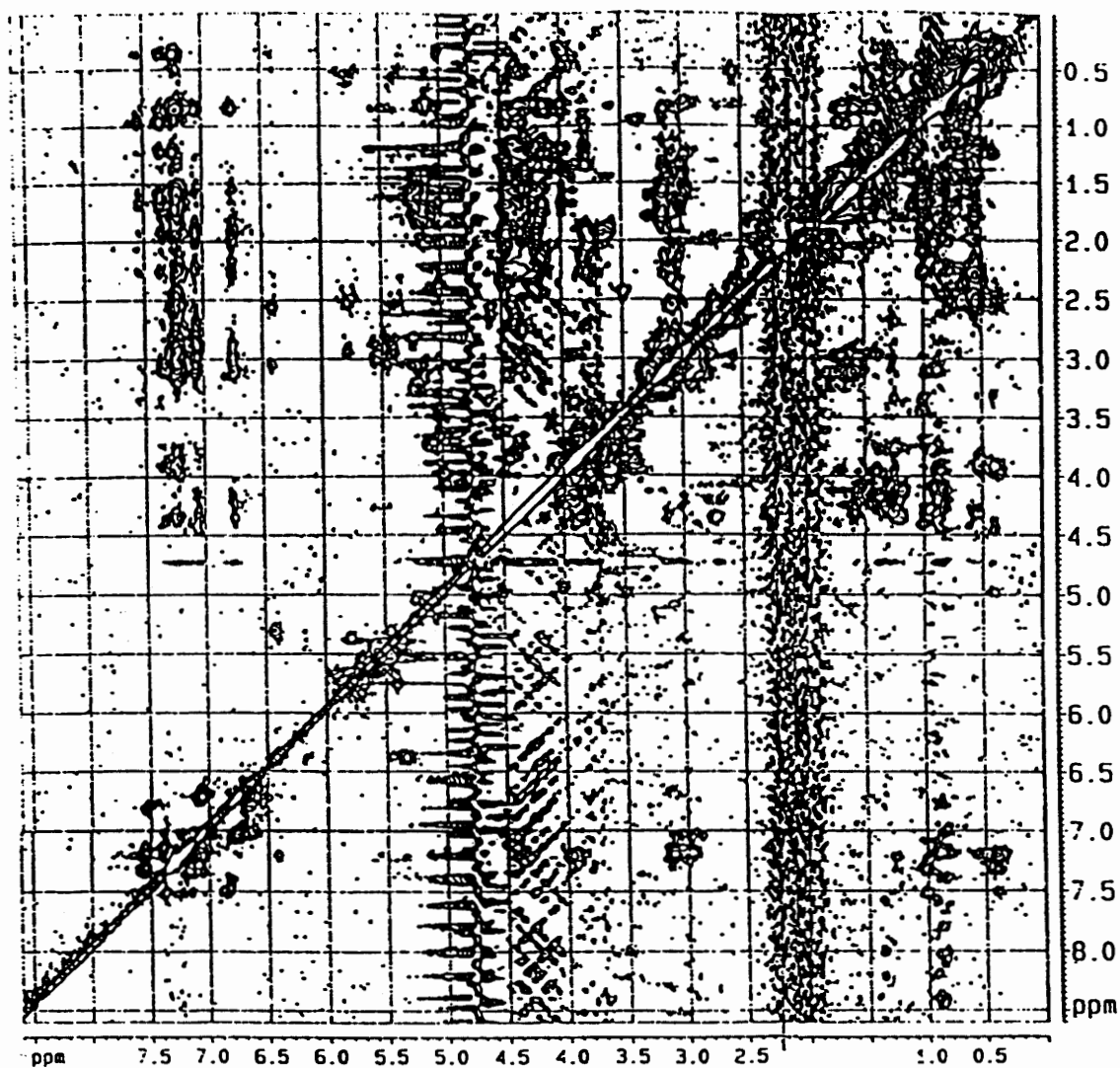


Figure 16: 2D NOESY spectra of 1 mM NP-I without acetonitrile in D<sub>2</sub>O at 25 °C and pH 6.0. The residual H<sub>2</sub>O resonance at 4.75 ppm was minimized by presaturation. The carrier frequency for p11 detection sequence was place at 2.01 ppm.

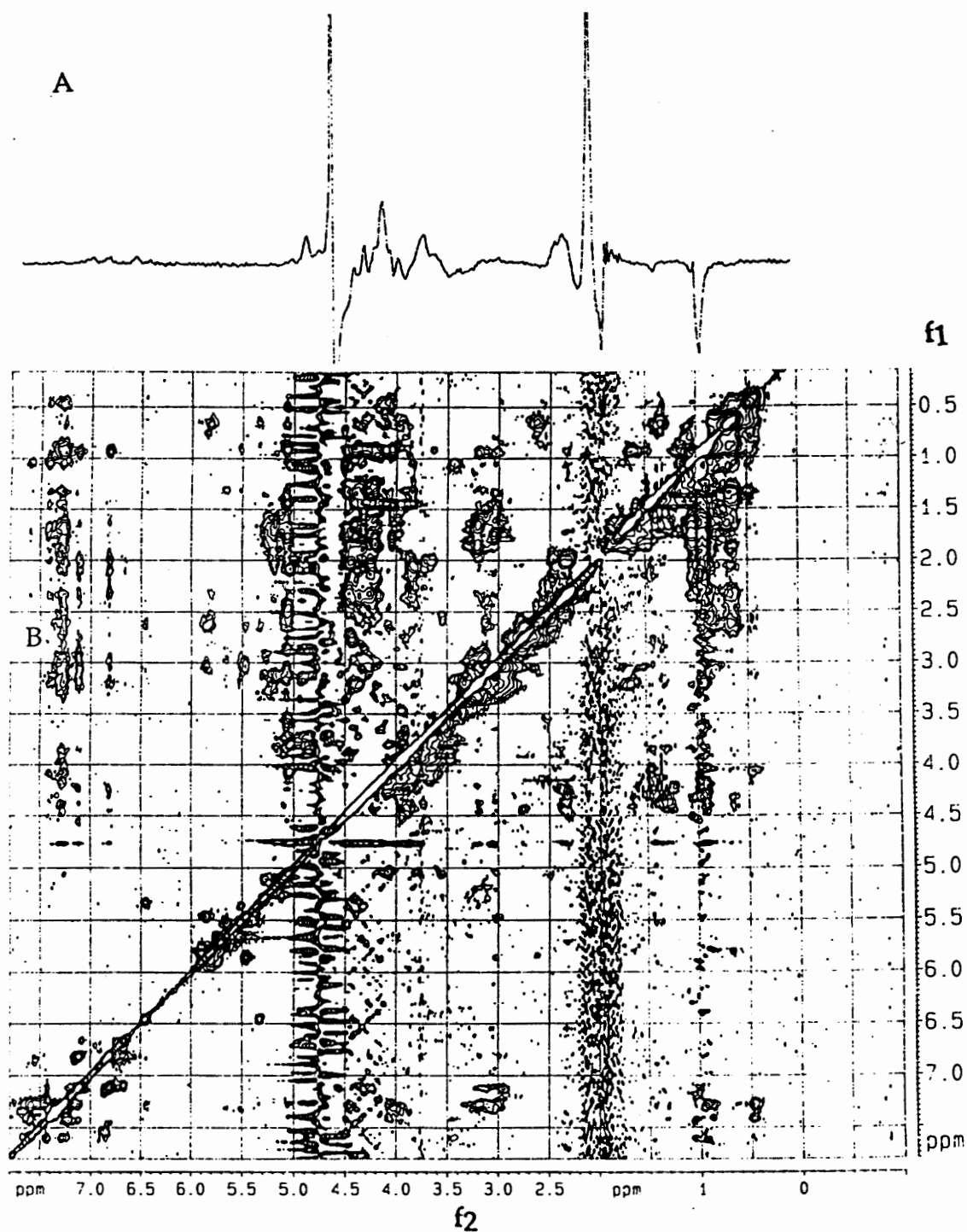


Figure 17: (A) Corresponding cross section parallel to  $f_2$  axis taken at the frequency of acetonitrile resonance at 1.95 ppm. (B) 2D NOESY spectra of 1 mM NP-I with 10% acetonitrile- $d_3$  in  $\text{D}_2\text{O}$  at 25  $^\circ\text{C}$  and pH 6.0.



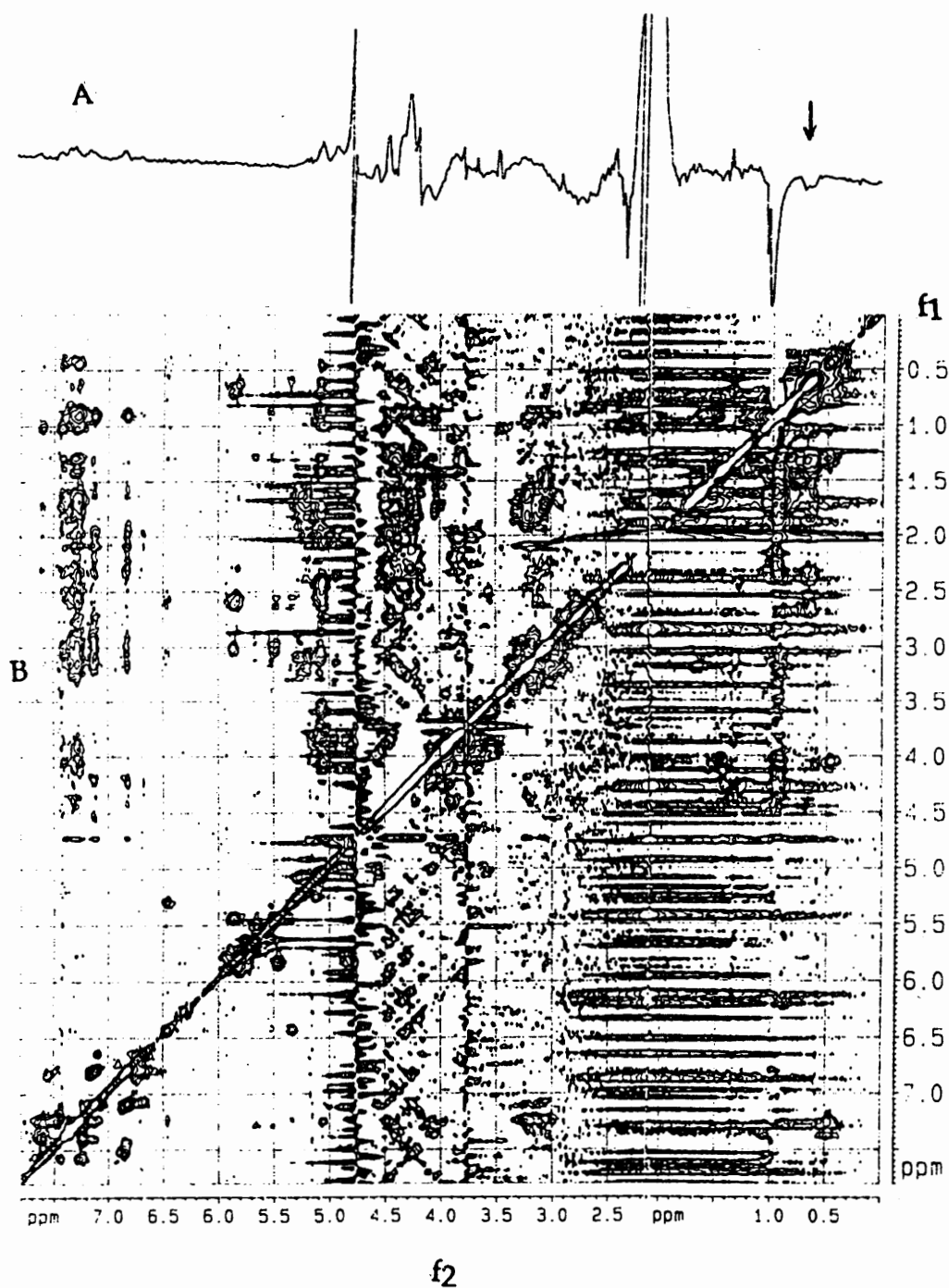


Figure 18: (A) Corresponding cross section parallel to  $f_2$  axis taken at the frequency of acetonitrile resonance at 1.95 ppm. (B) 2D NOESY spectra of 1 mM NP-I with 10% acetonitrile in  $D_2O$  at 25  $^{\circ}C$  and pH 6.0.

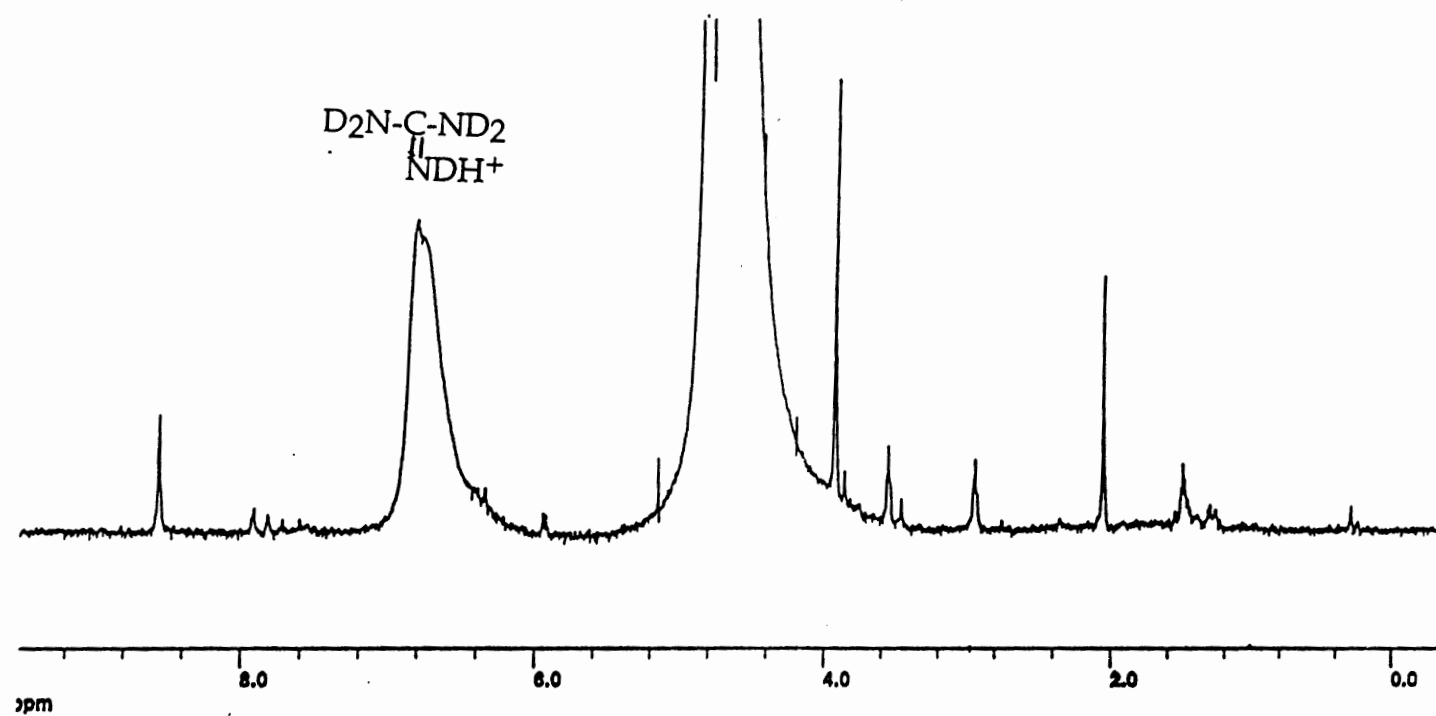


Figure 19: 1D spectra of guanidine monohydrochloride at 25 °C and pH 6.0.

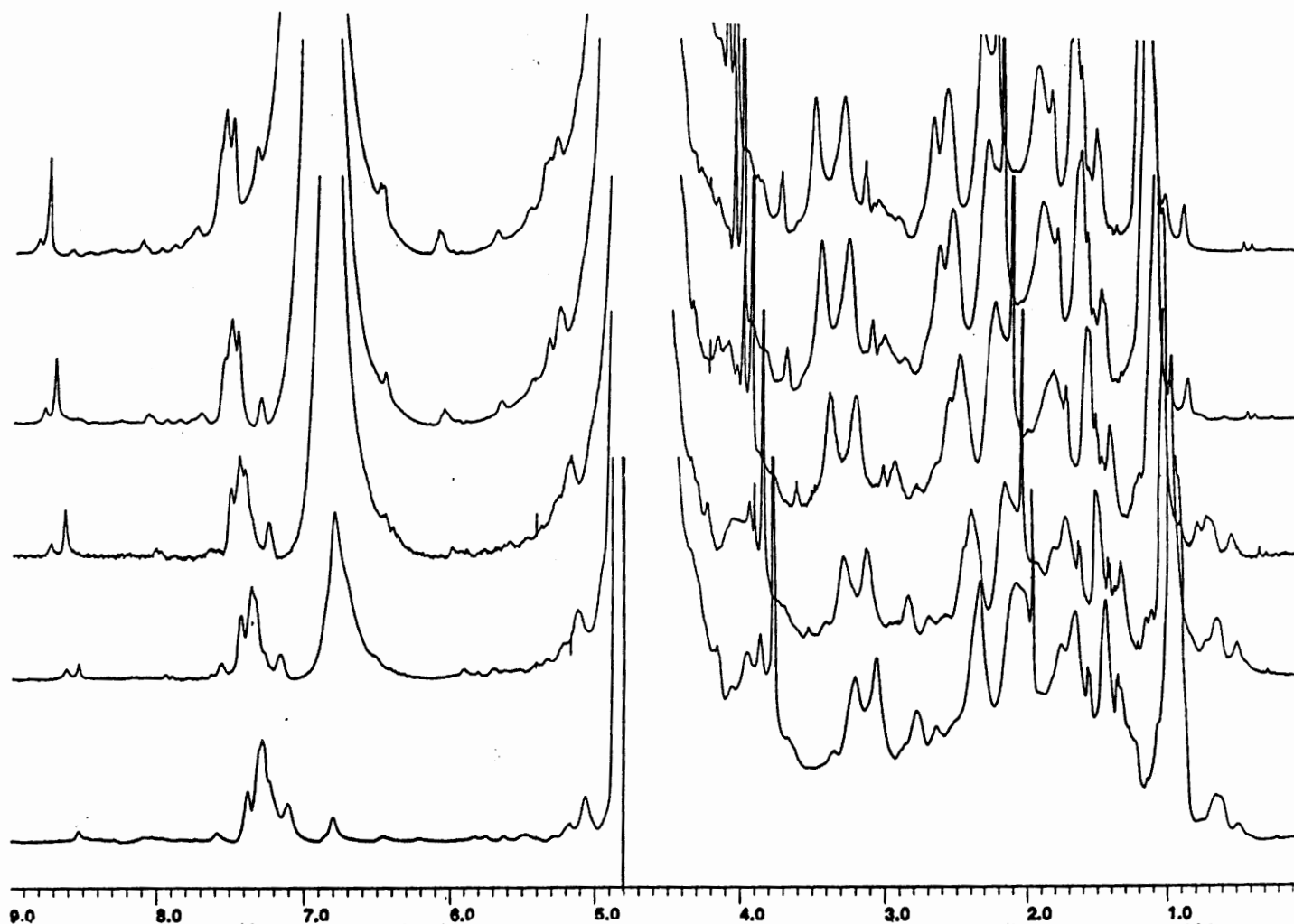


Figure 20: 1D spectra showing Gnd·DCl titration of NP-I in D<sub>2</sub>O. Spectra were obtained at 25 °C and pH 6.0 from 0 M Gnd·DCl (bottom), 1 M, 3 M, 5 M and 7 M (top).

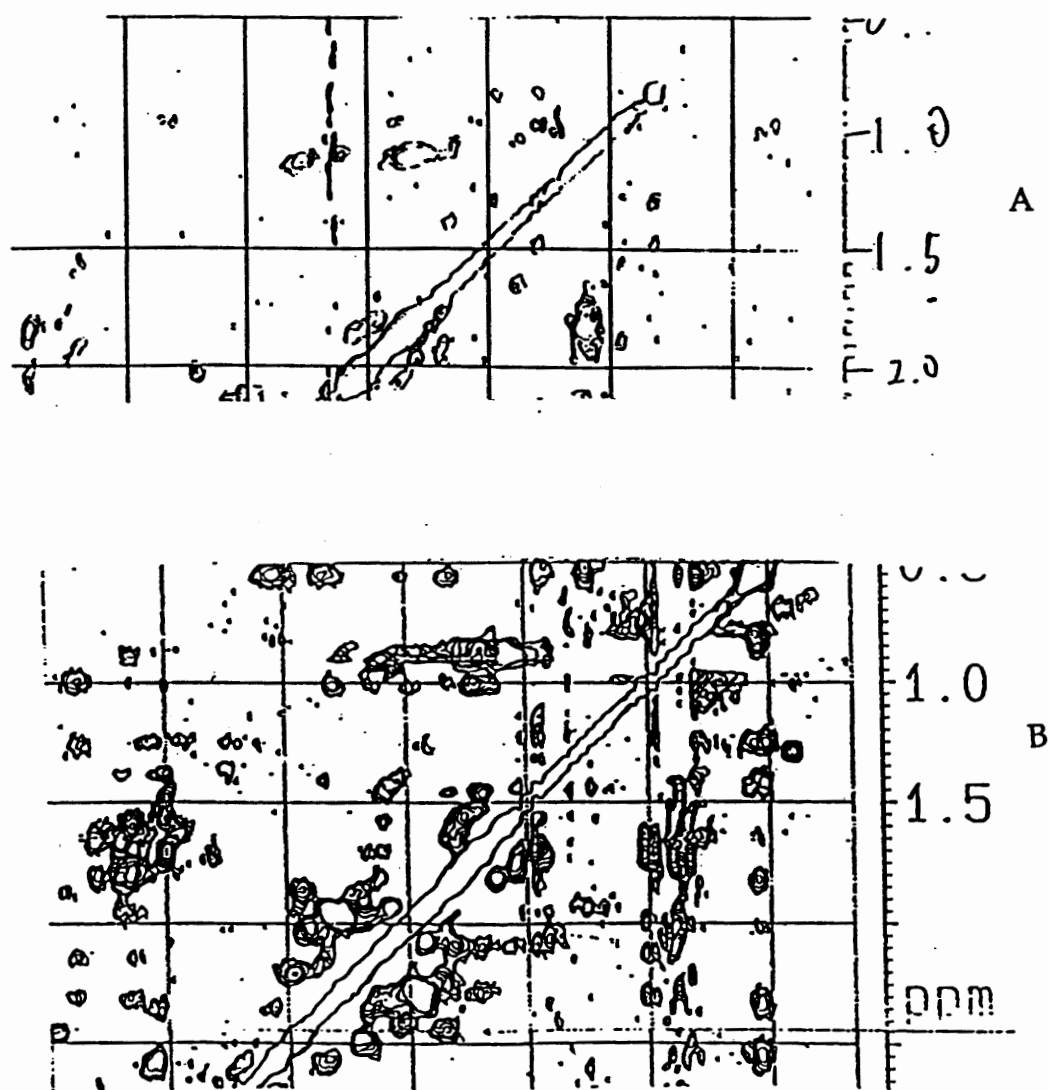


Figure 21: (A) 2D NOESY spectra of 1 mM NP-I at 25  $^{\circ}\text{C}$  and pH 6.0. (B) 2D NOESY spectra of NP-I in 7 M Gnd·DCI at 25  $^{\circ}\text{C}$  and pH 6.0.

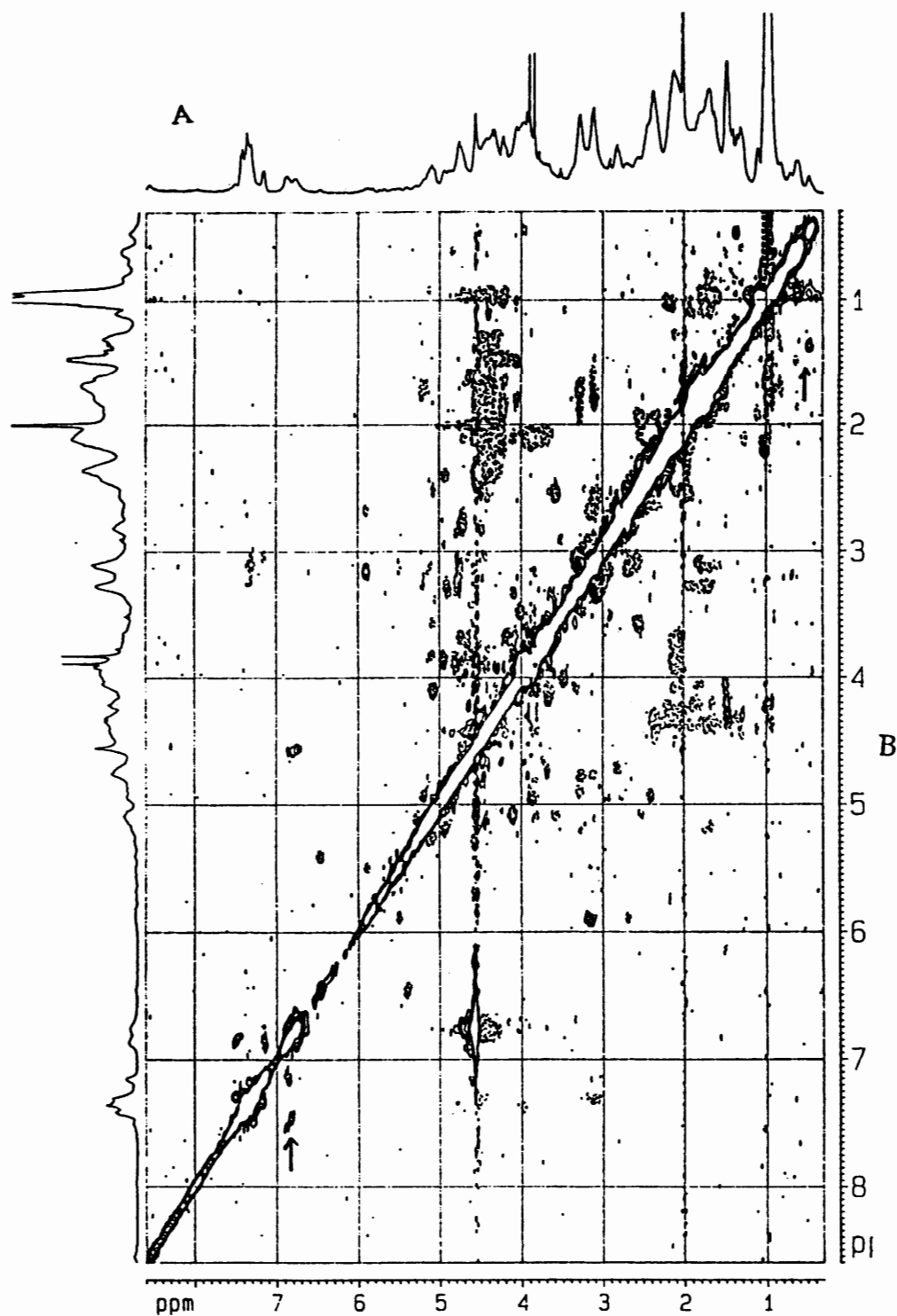


Figure 22: ROESY of NP-I in 2.5 M Gnd-DCI. (A) The 1D spectrum shown directly above the ROESY spectrum (B). Spectra were obtained at 45 °C and pH 6.0. Dotted cross-peaks represent NOE peaks. Solid-line cross-peaks are exchange peaks.

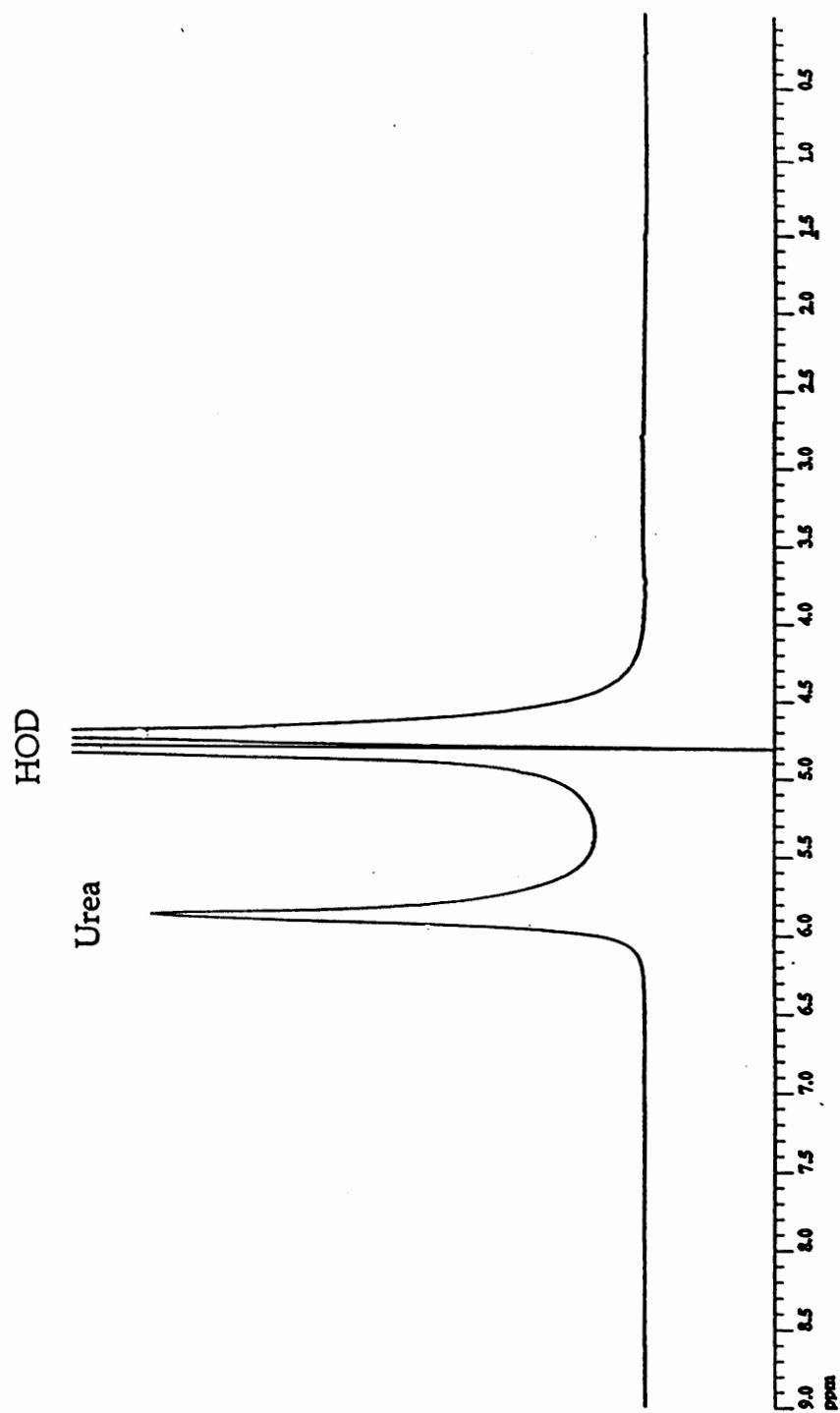


Figure 23: Spectra of urea in D<sub>2</sub>O at 25 °C and pH 6.0.

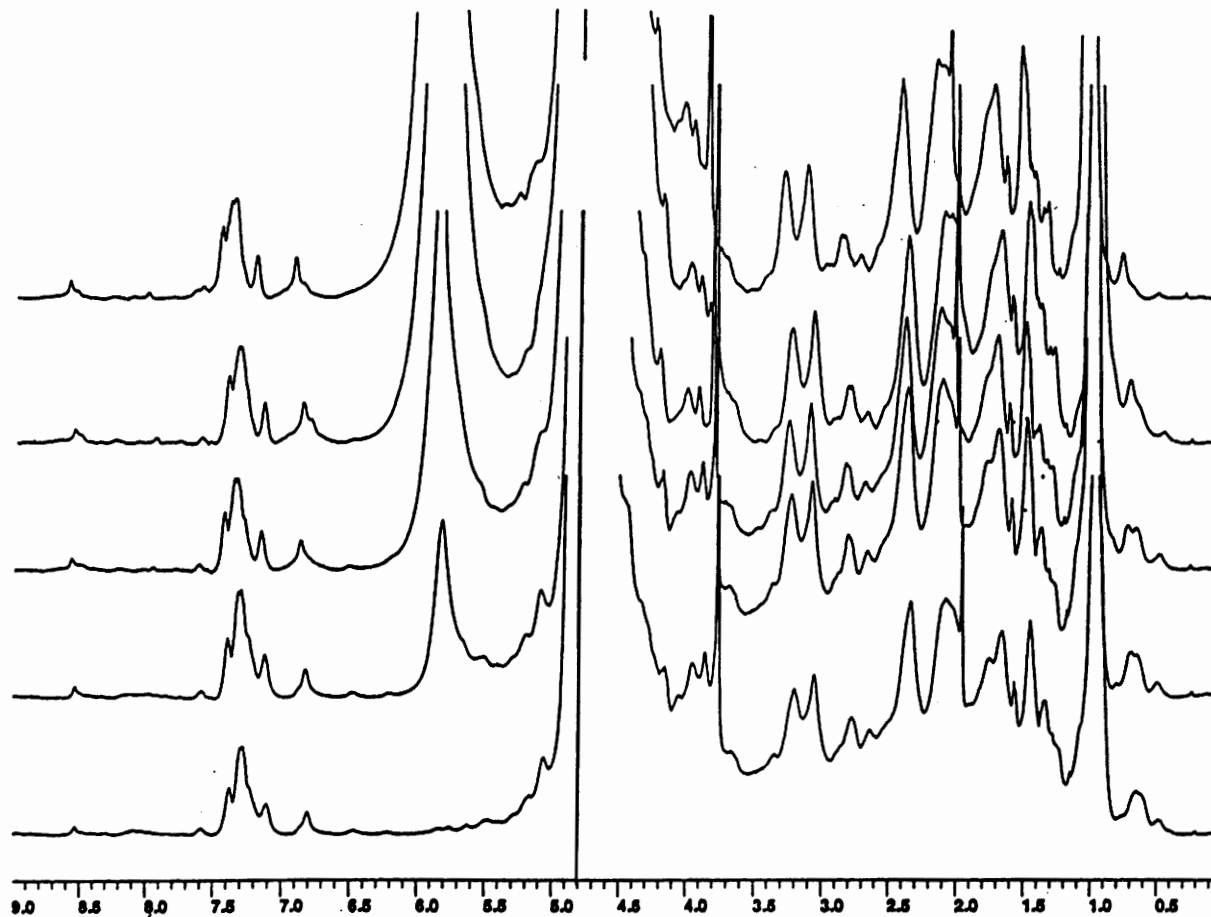


Figure 24: 1D spectra showing urea titration of NP-I in D<sub>2</sub>O. Spectra were obtained at 25 °C and pH 6.0 from 0 M urea (bottom), 1 M, 3 M, 5 M, and 7 M (top).

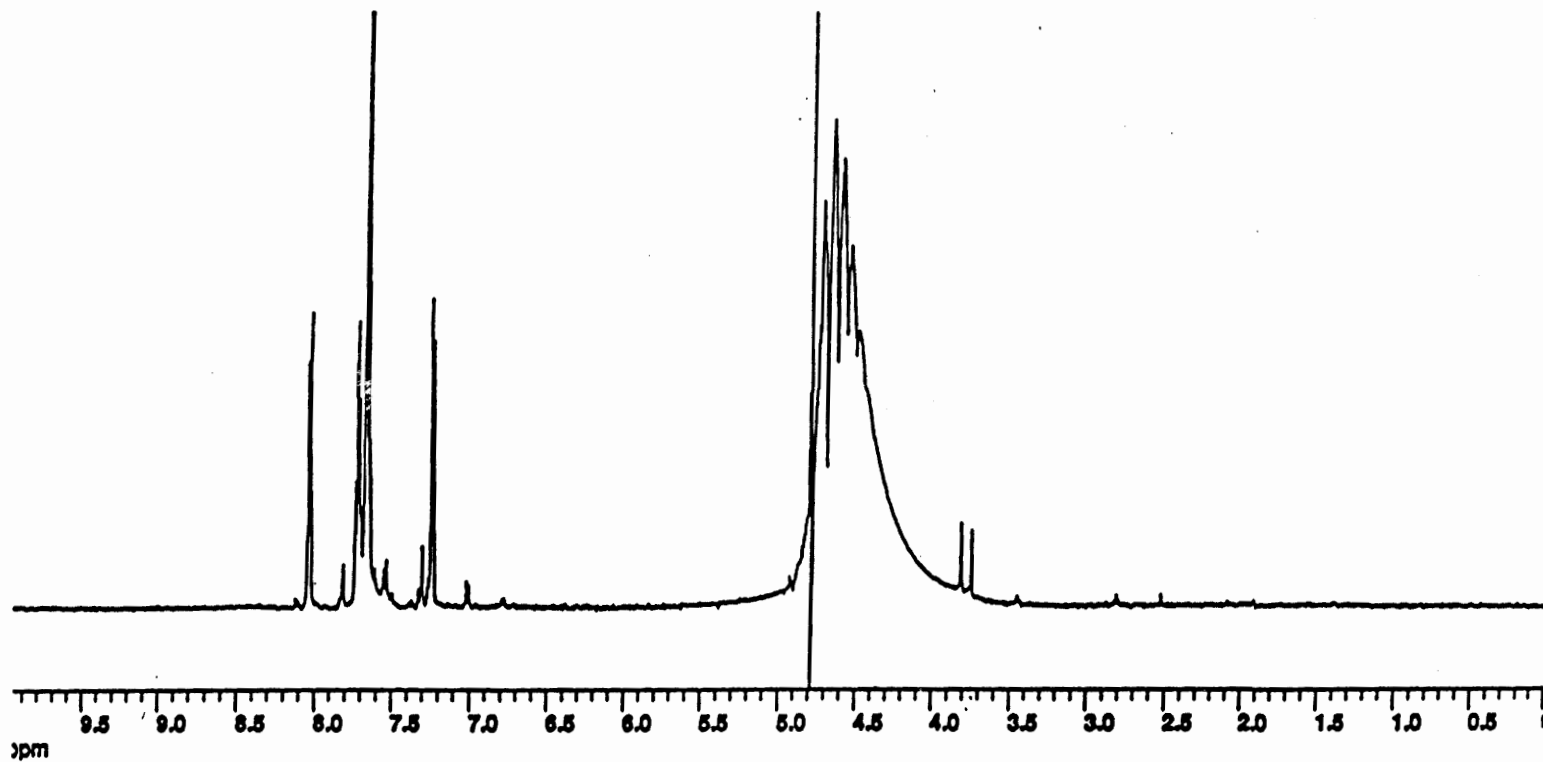


Figure 25: 1D spectrum of 1 mM bromophenol blue at 25 °C and pH 2.3.



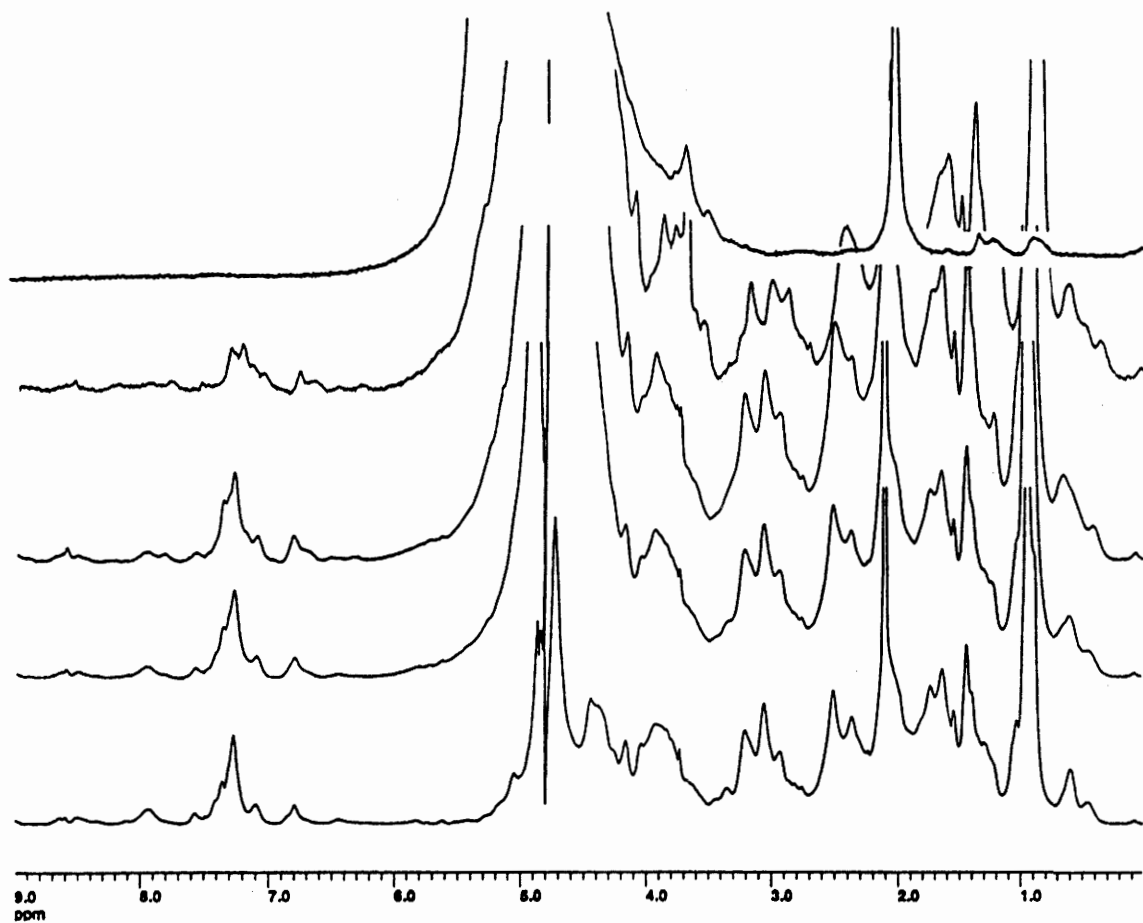


Figure 26 1D spectra showing bromophenol blue titration of NP-I in D<sub>2</sub>O at 25 °C and pH 2.3. NP-I concentration is 2.7 mM. The molar ratio of NP-I to dye is from 1:0 (bottom), 1:0.4, 1:1, 1:2.5 to 1:4.

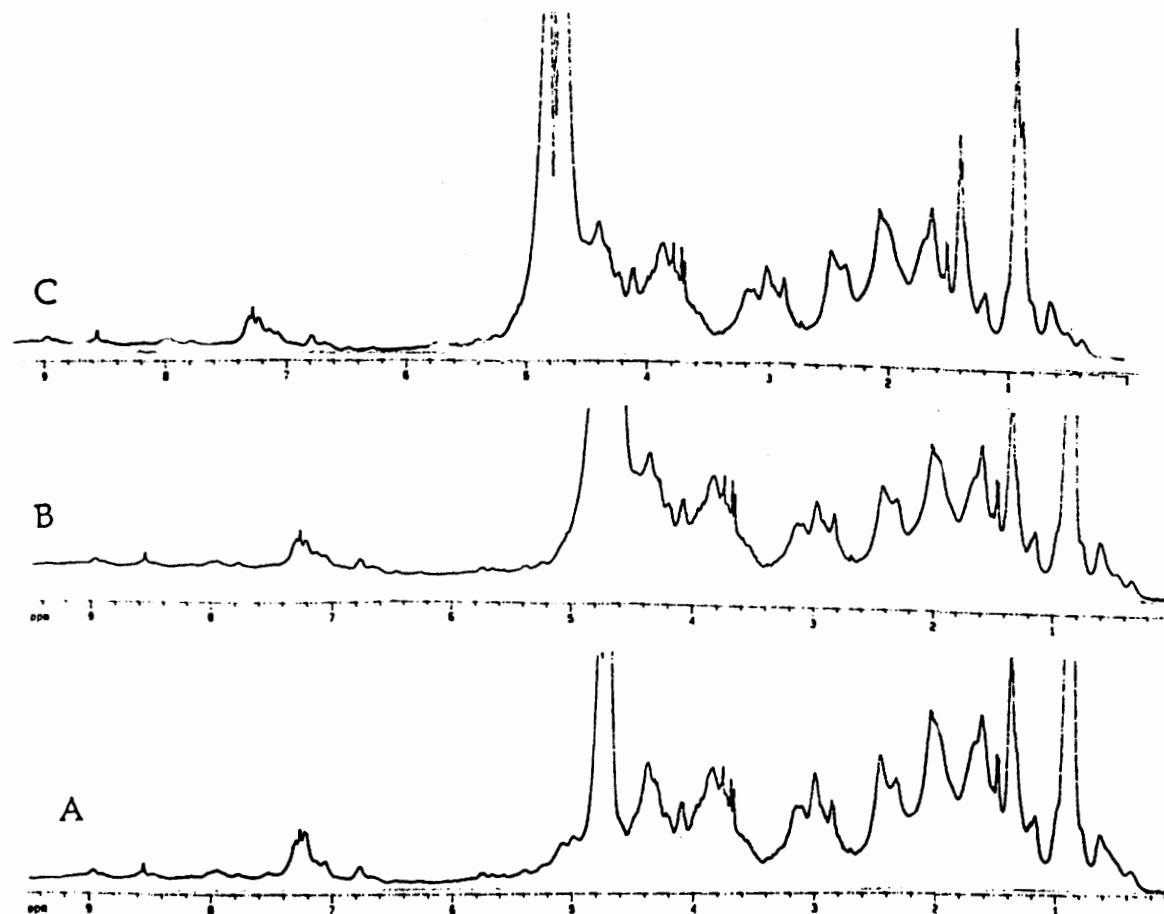


Figure 27: (A) 1D spectrum of dye-free NP-I in D<sub>2</sub>O at 25 °C and pH 2.3. The concentration of NP-I is 0.2 mM. (B) 1D spectra of titration of bromophenol blue of NP-I. Spectra were obtained at 25 °C and pH 2.3 at 1:1 and 1:2 (top) of NP-I to dye molar ratio.

## CHAPTER 4

### CONCLUSION

NP self-associates into dimer at high concentration. This self-association involves interfacial  $\beta$ -structure/ $\beta$ -structure contact. There are two methods to lower the NP dimerization constant. One is lower the concentration of NP. The other is modulate the solvent system. Because the signal-to-noise ratio would be too small at the extremely low concentration of NP, and NP self-associates into dimer at the concentration found in biological condition in pituitaries, it is necessary to use the second method to break the NP dimerization if monomer is to be studied.

NMR was used to find specific identities of resonances which are perturbed by cosolvent change. Cosolvent effects were investigated by conventional 1 D, 2 D NOESY, and 2 D ROESY NMR.

A set of cosolvents was titrated into the regular deuterium solvent ( $D_2O$ ). The dimer-dissociation-sensitive resonances were monitored by 1 D NMR spectra. It was found that among the organic cosolvents used, only acetonitrile and propionitrile be able to lower the NP dimerization constant significantly. This is presumably because they disturbed the hydrophobic monomer-monomer interface and destabilized the  $\beta$ -sheet hydrogen bond contact. The interaction between acetonitrile and the monomer-monomer interface was further studied by 2 D NOESY spectra.

From the observations, it was concluded that acetonitrile may bind to the NP-I monomer interface residues to cause the dissociation of dimer.

The effects of the protein denaturing reagents, guanidine·DCl and urea, were investigated by 1 D NMR, 2 D ROESY, and 2 D NOESY spectra. The results indicate that the denaturing reagents unfold NP before dimer dissociates.

The binding of bromophenol blue to NP was studied. But the NP-dye complex is not soluble enough to be studied by NMR, at least under the conditions used in this study.

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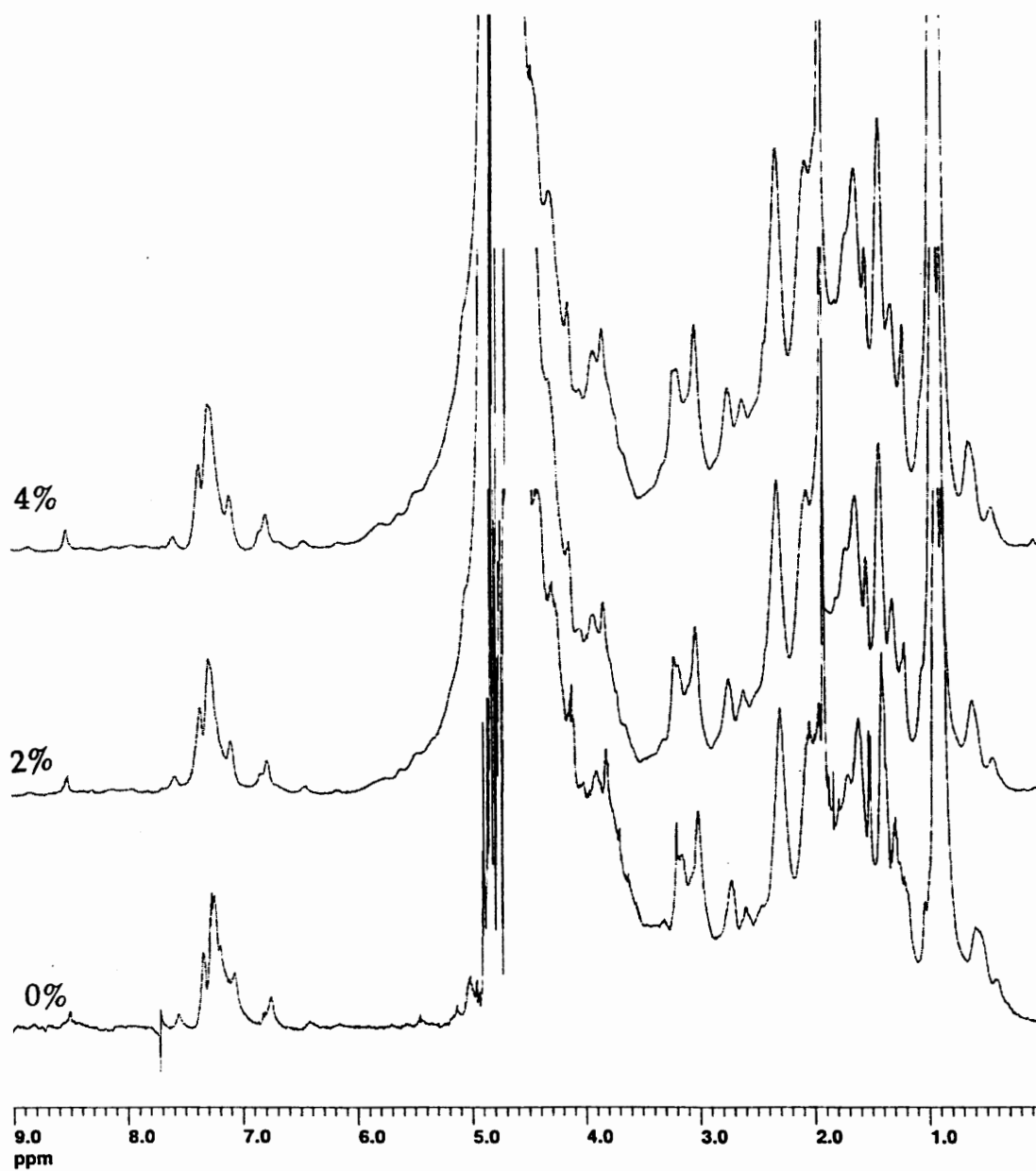
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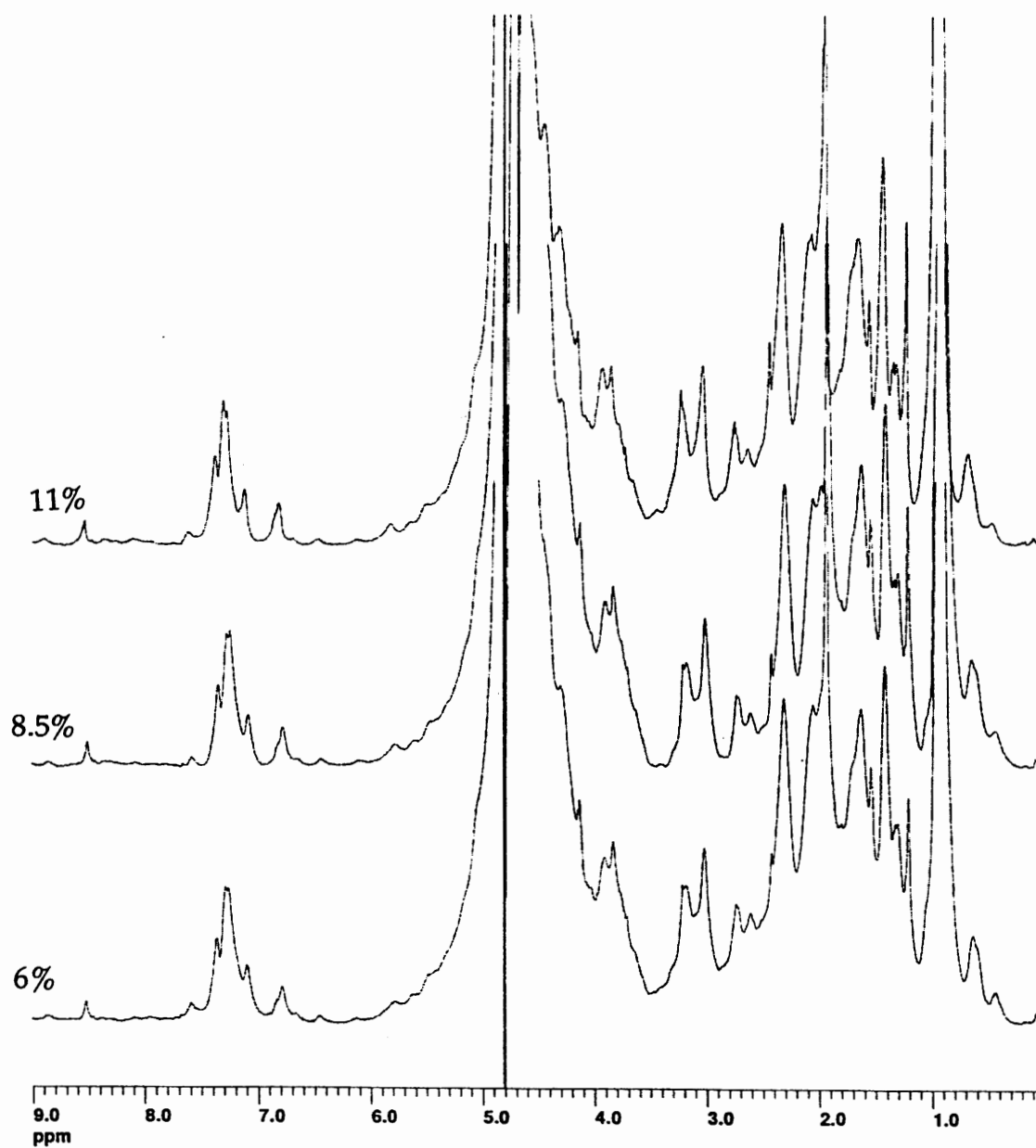
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## APPENDIX

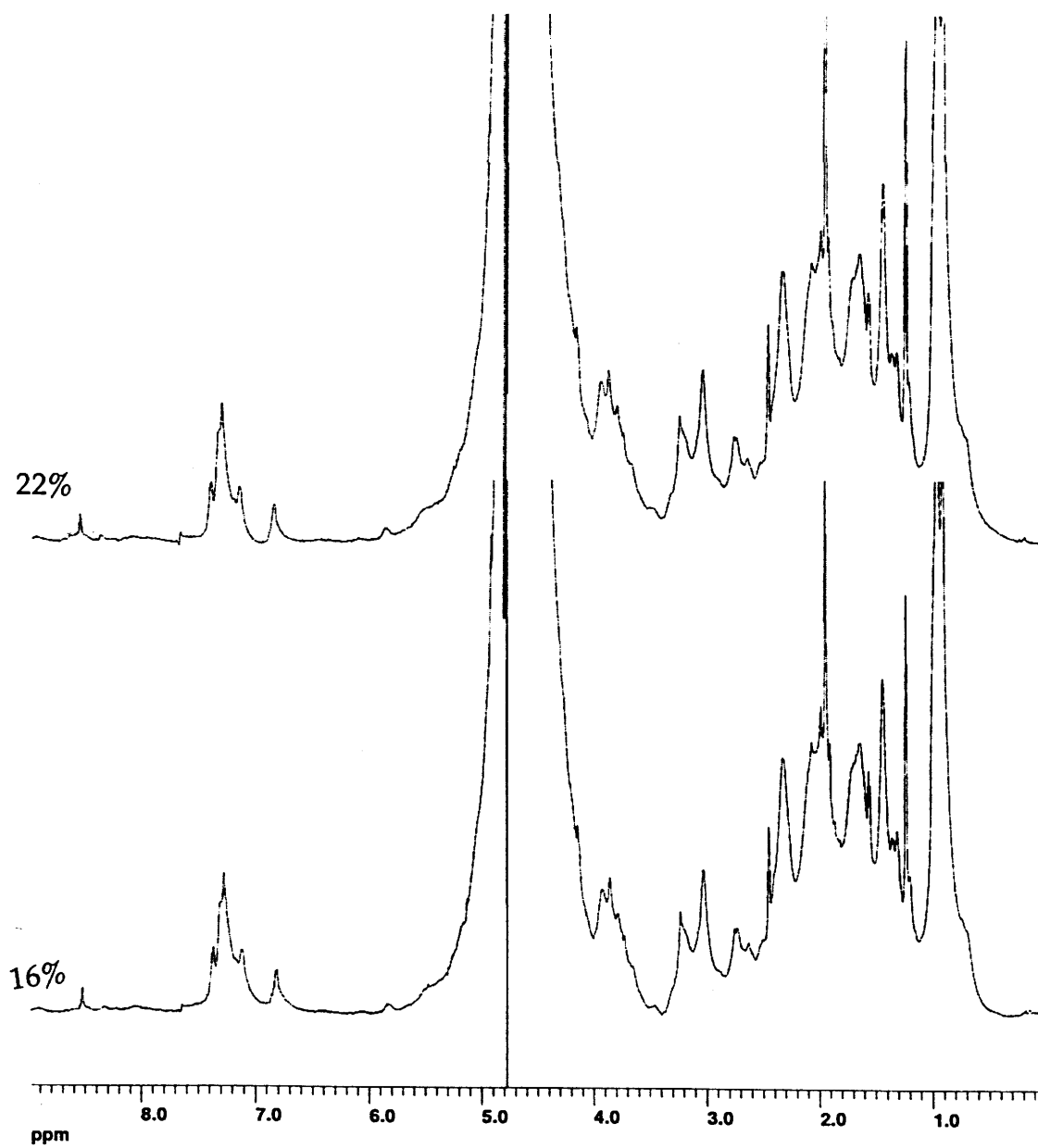
Full sets of 1 D NMR spectra of titration of NP-I with cosolvents at 25 °C, pH 6.0.



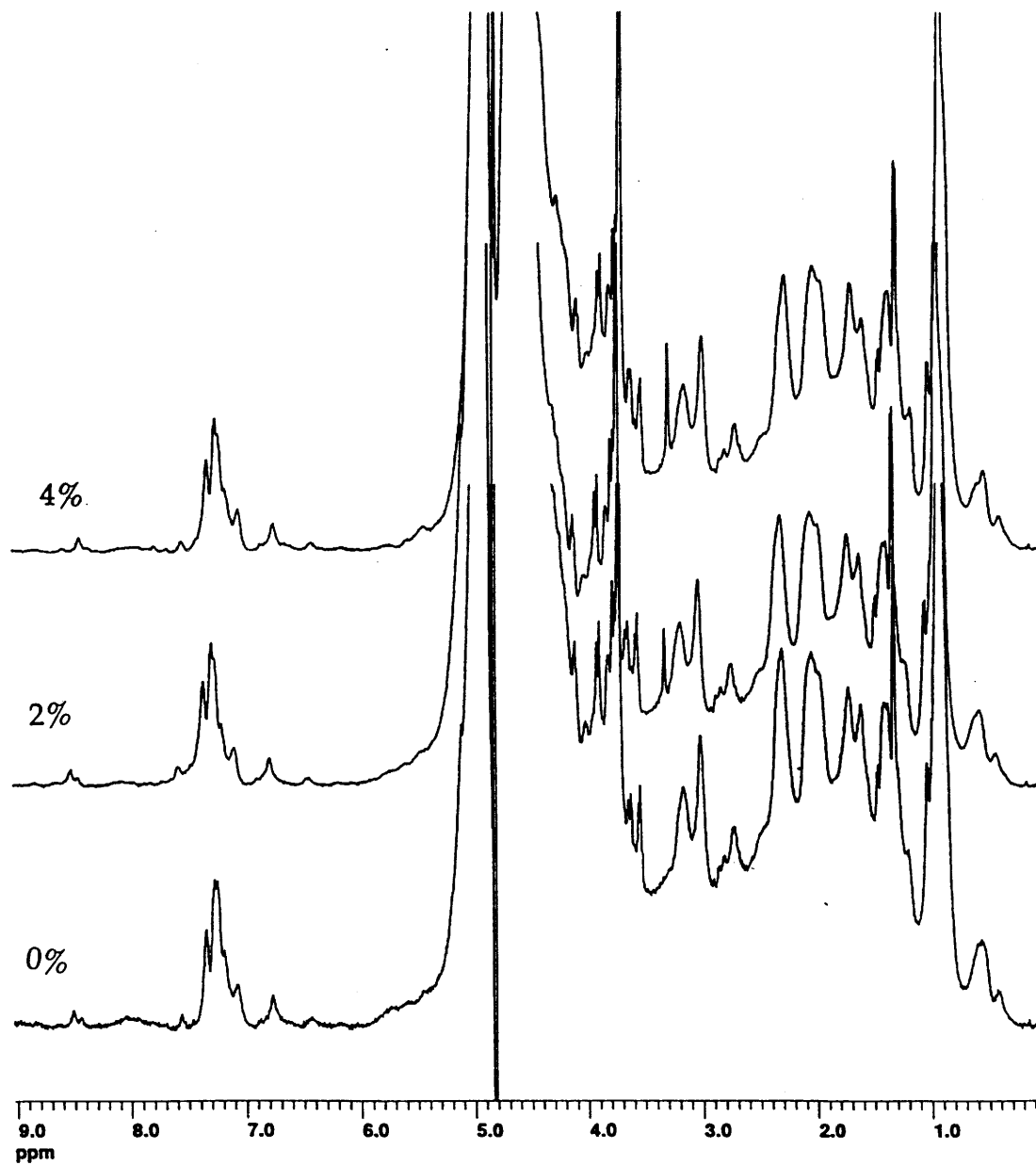
NP-I titrated with propionitrile



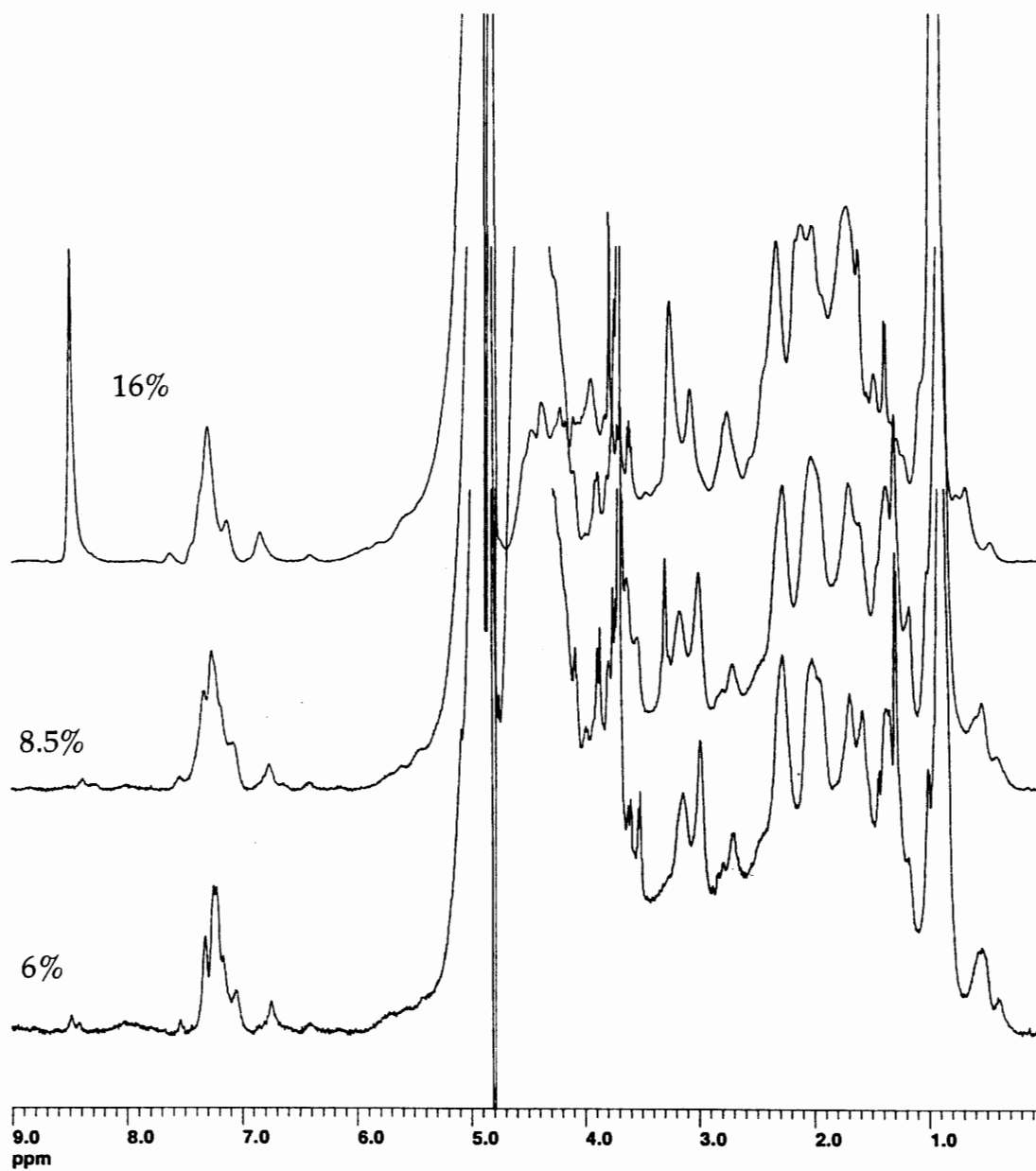
NP-I titrated with propionitrile



NP-I titrated with propionitrile



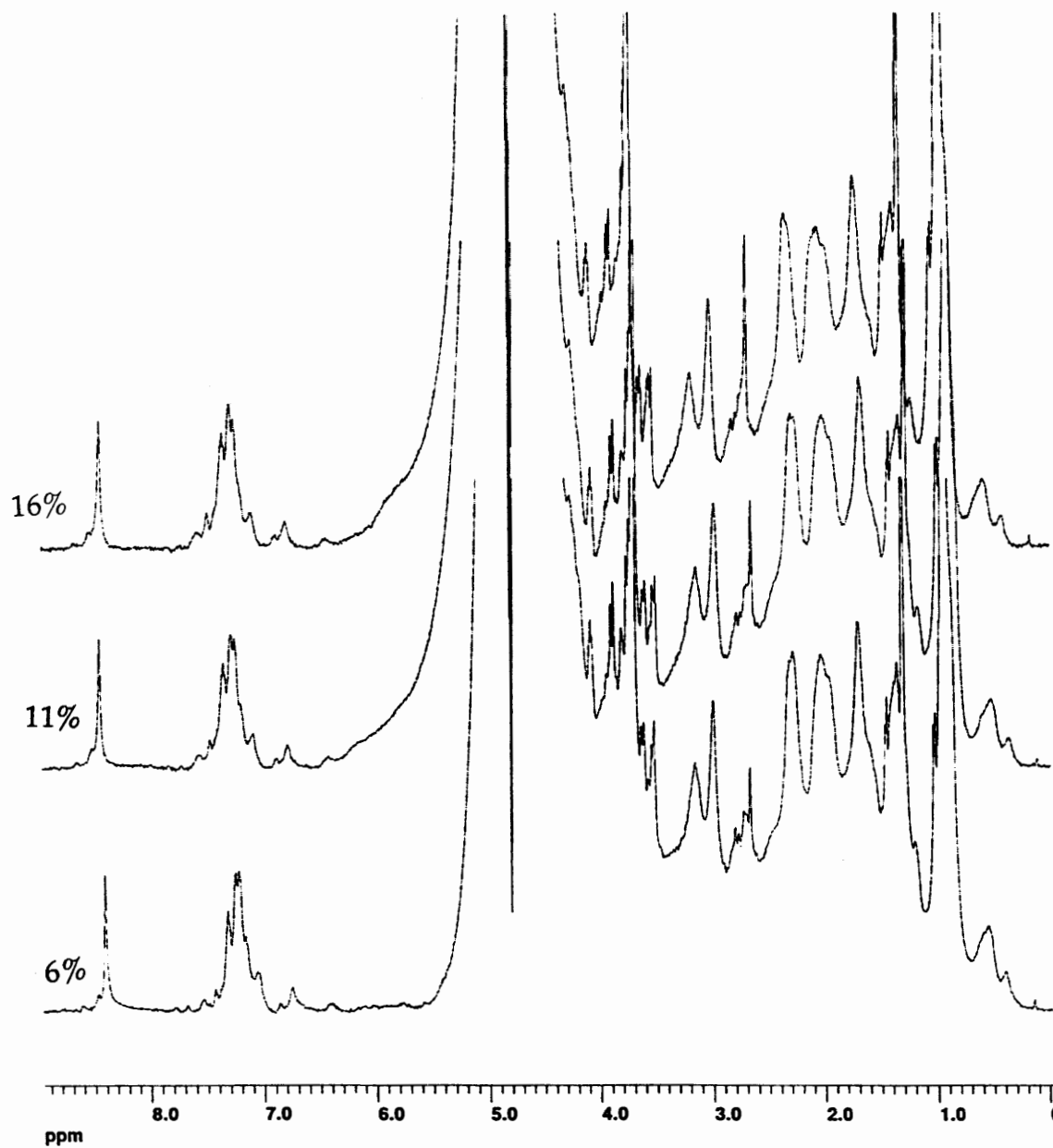
NP-I titrated with methanol



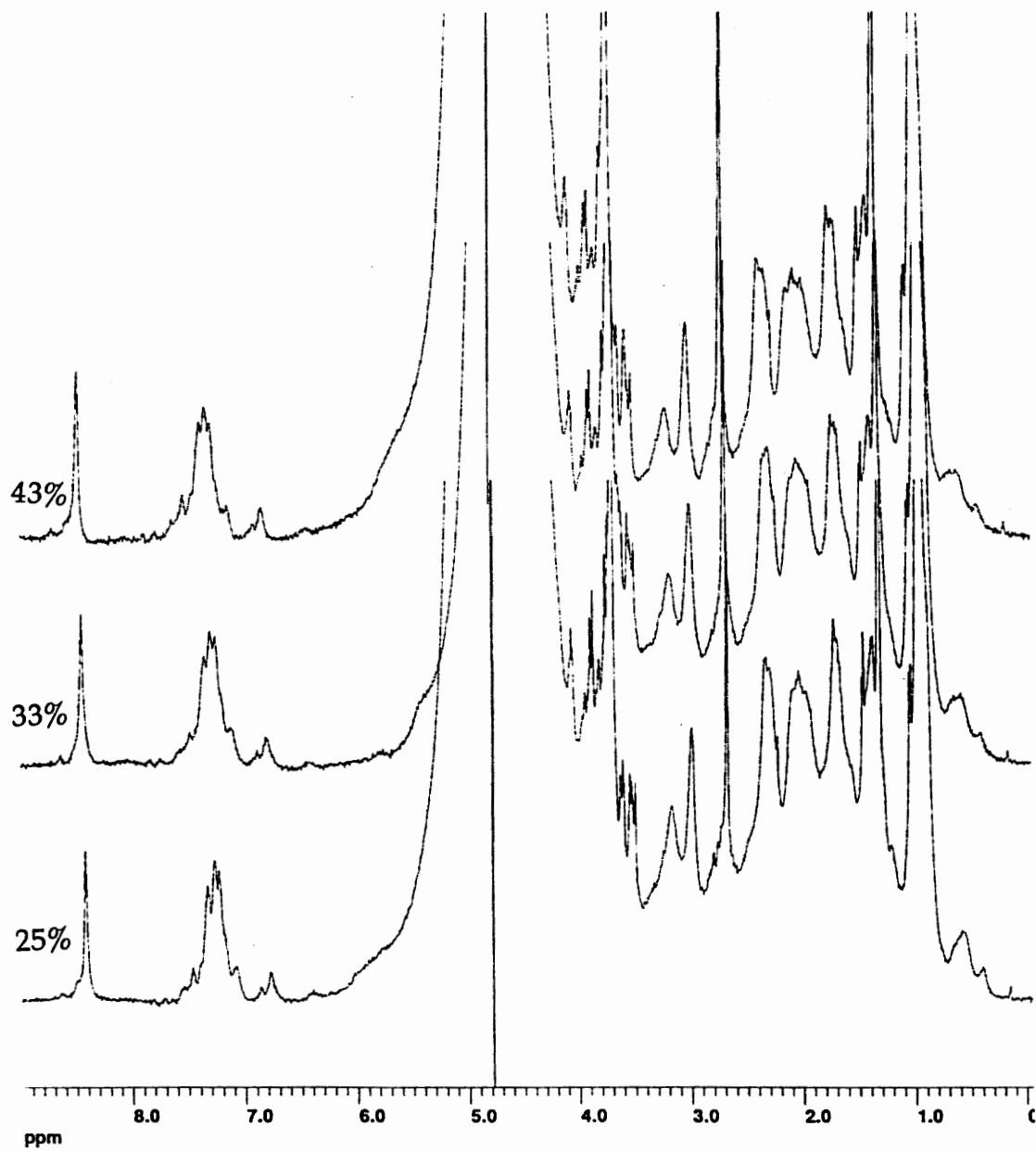
NP-I titrated with methanol

NP-I titrated with DMSO

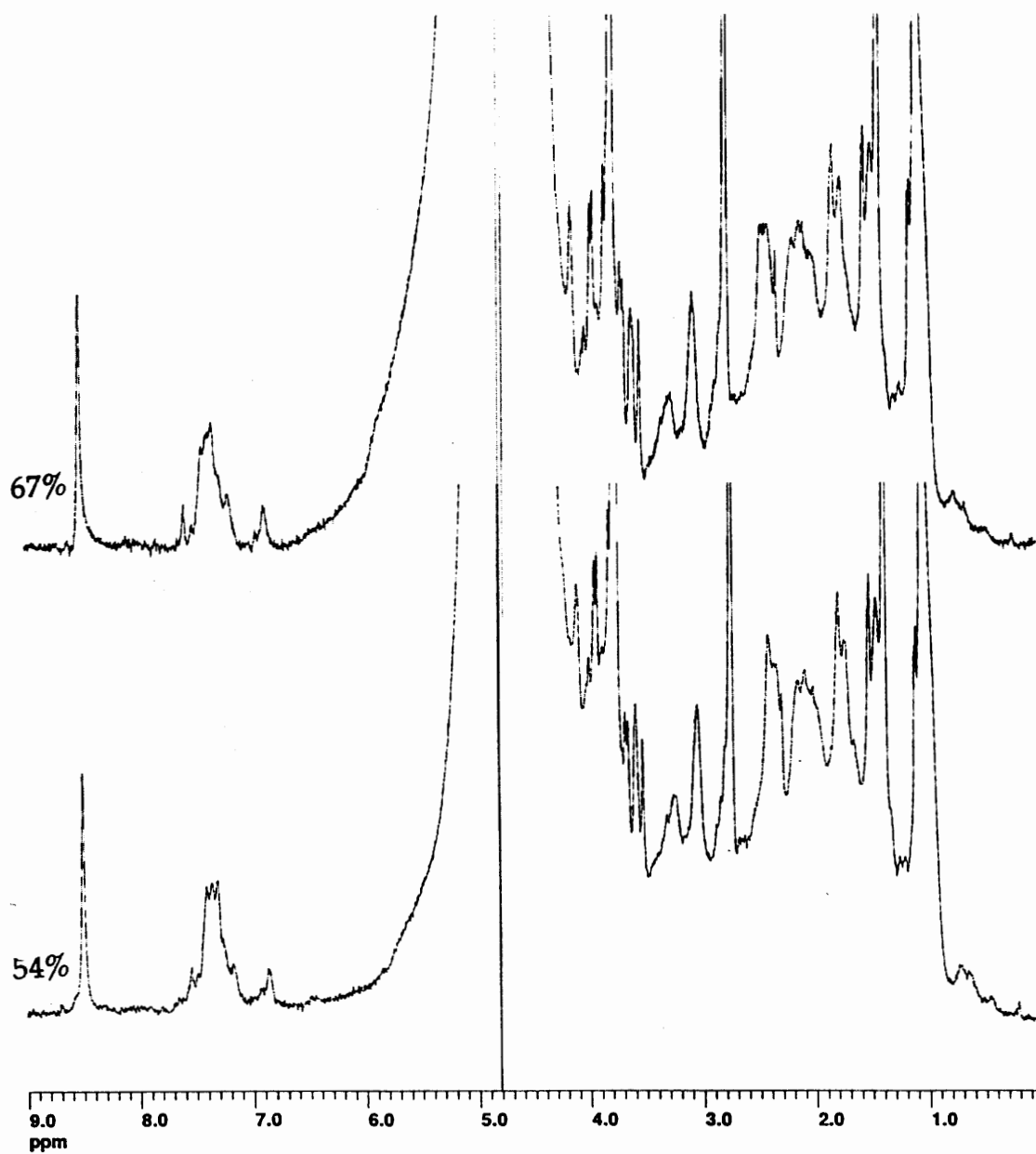




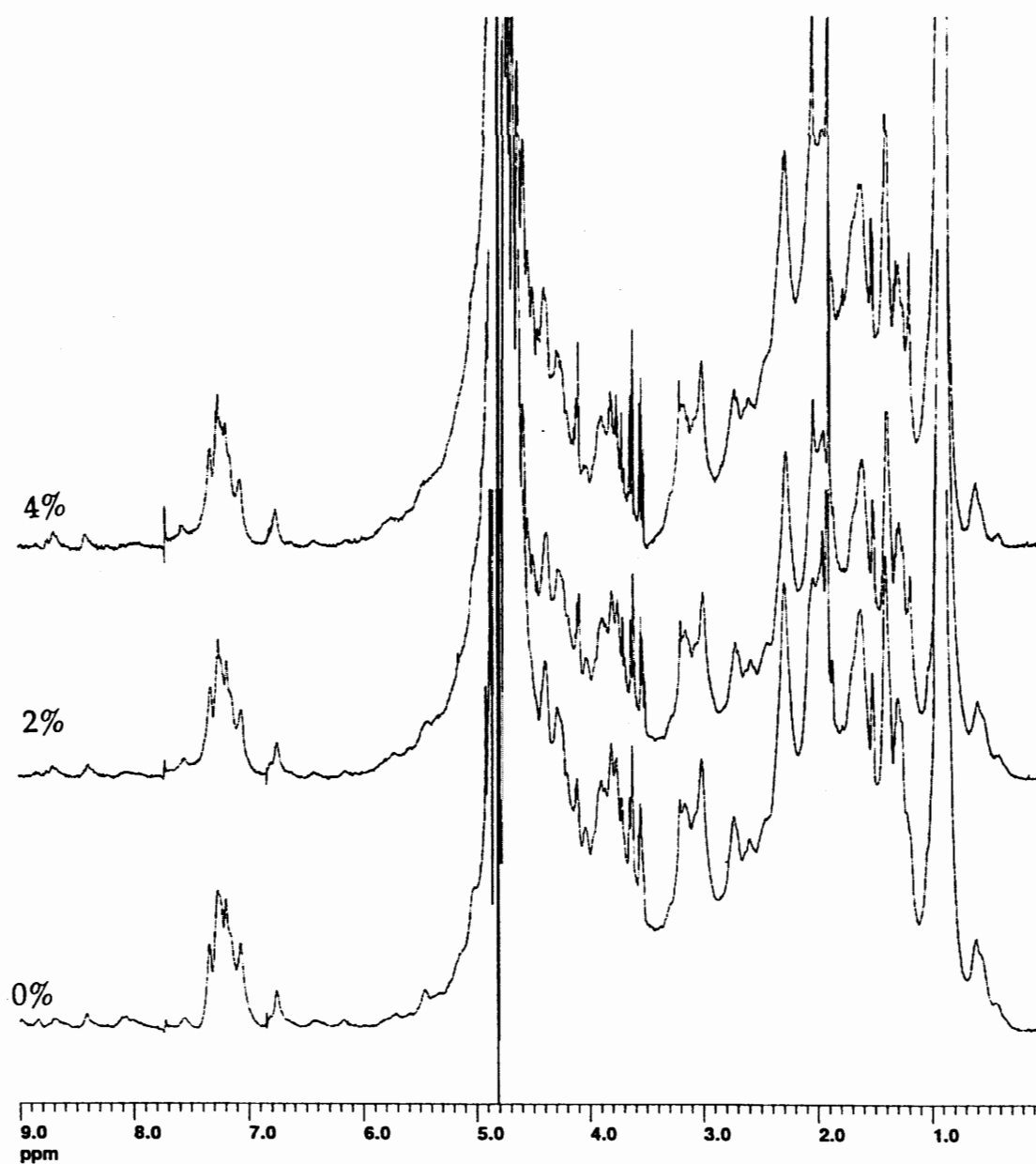
NP-I titrated with DMSO



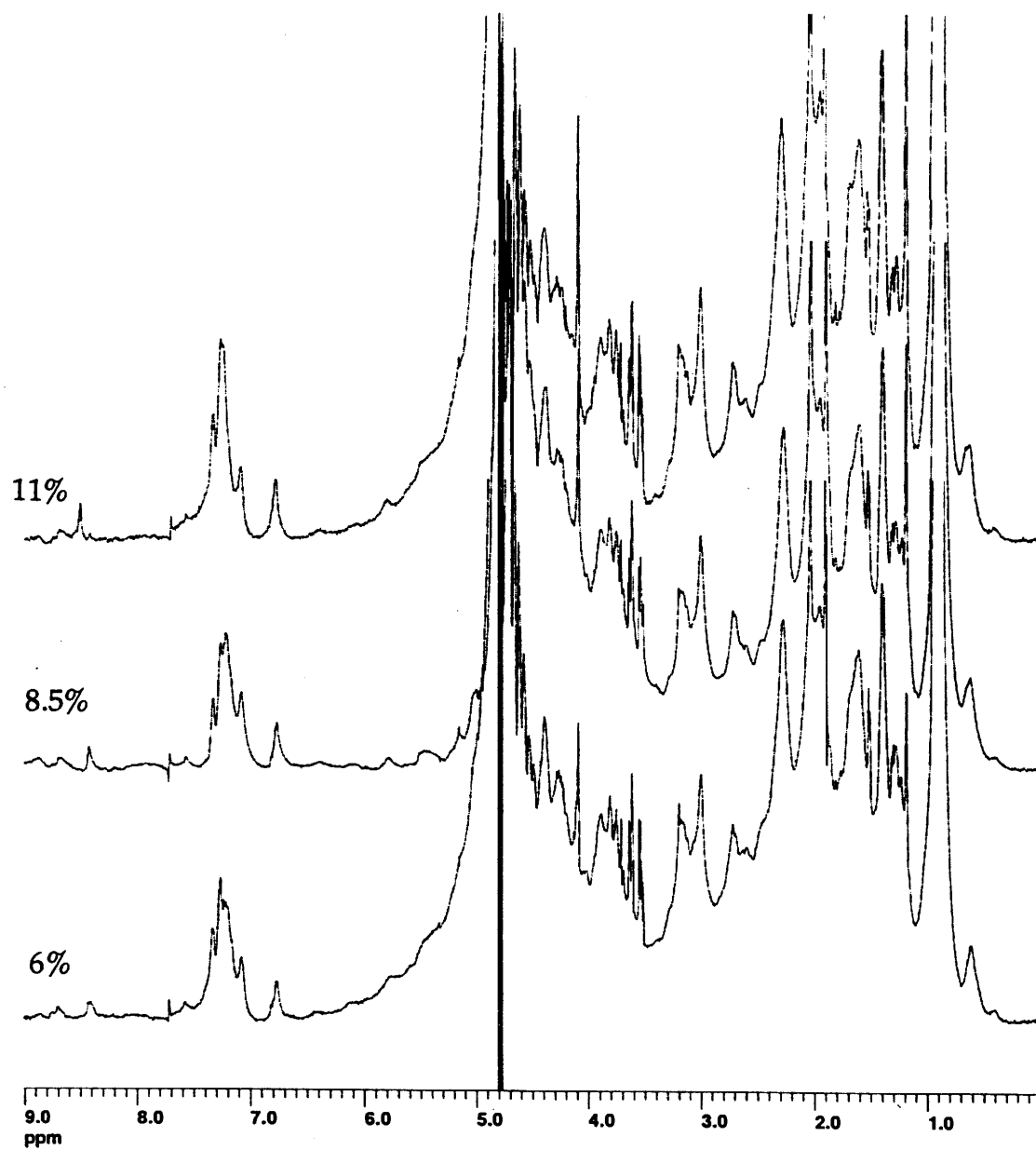
NP-I titrated with DMSO



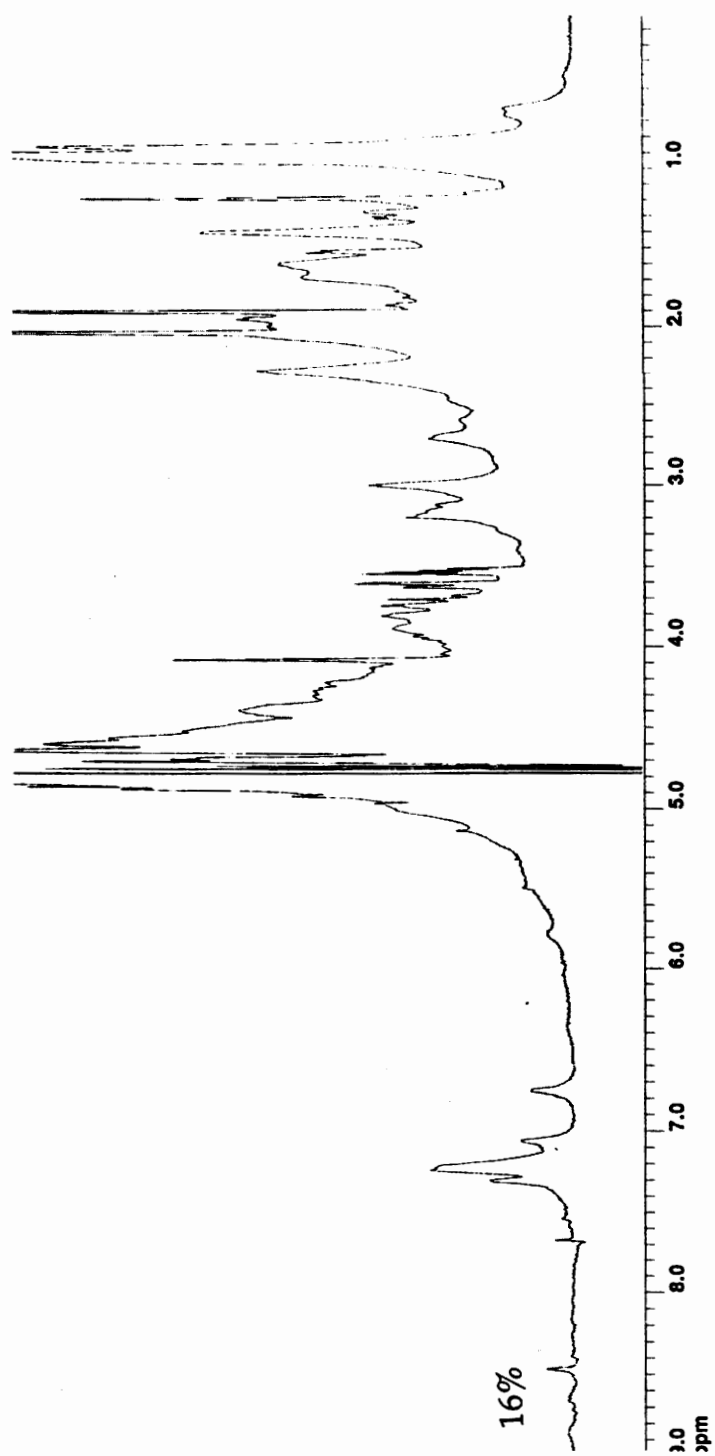
NP-I titrated with DMSO



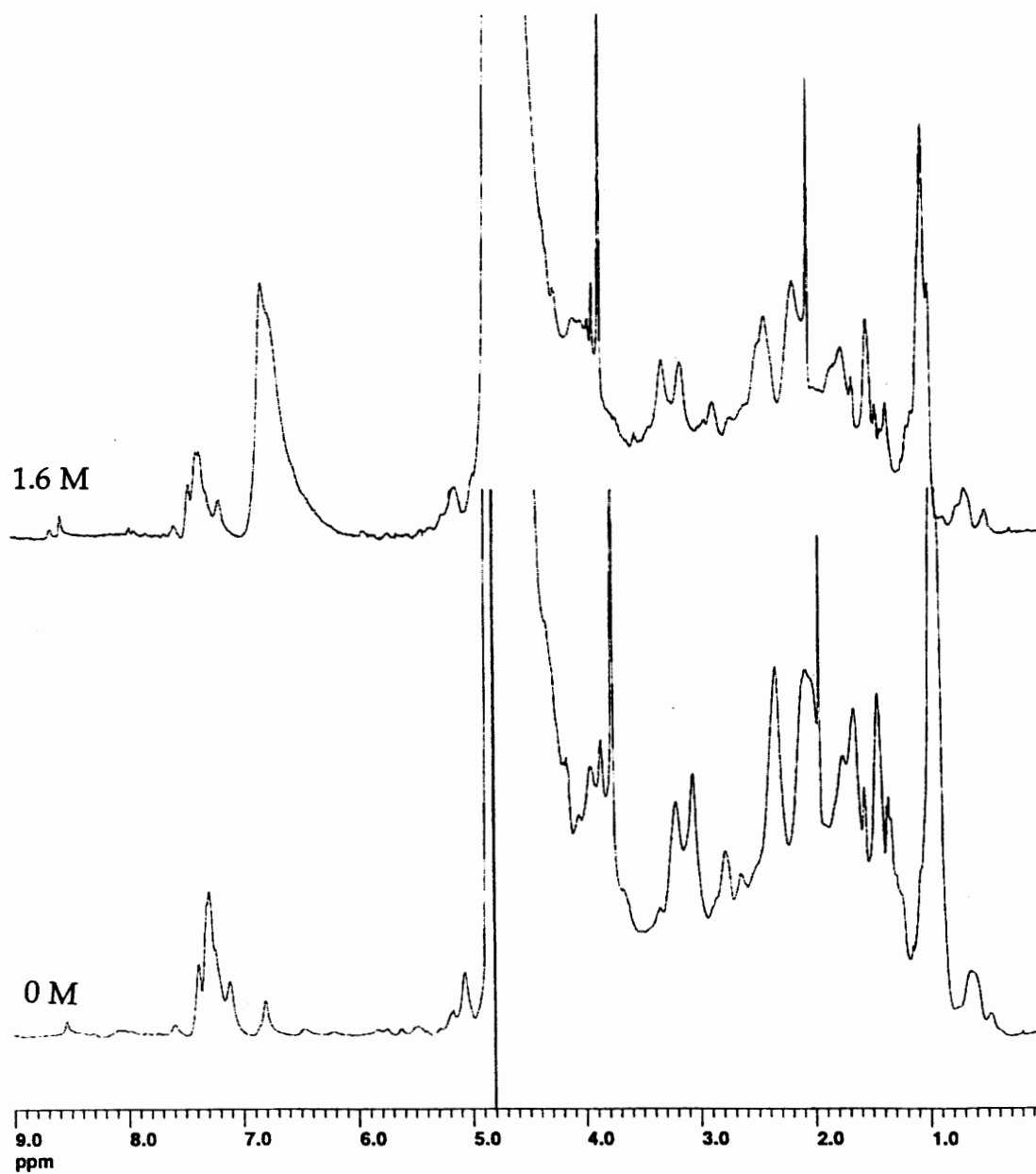
NP-I titrated with ethyl acetate



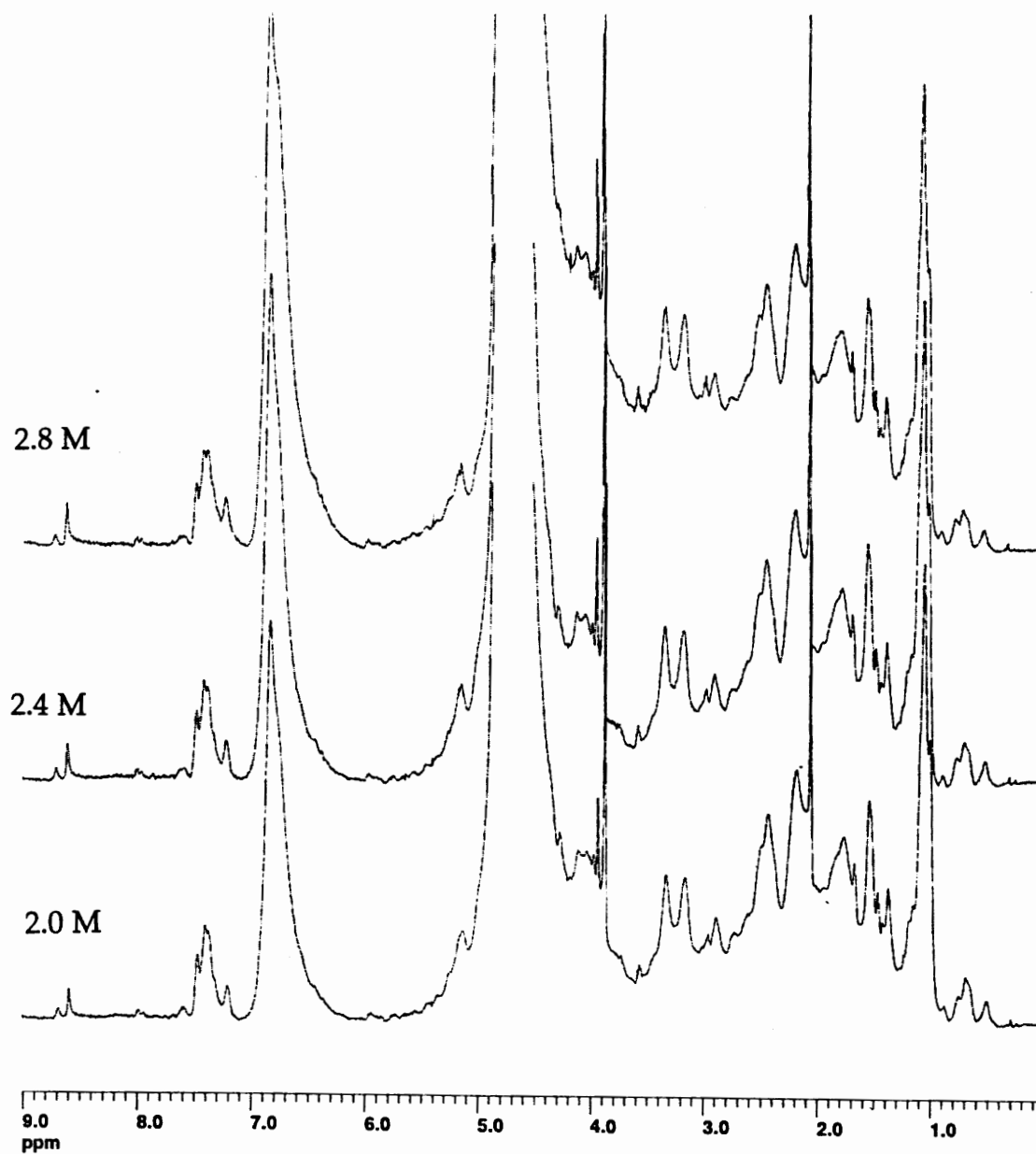
NP-I titrated with ethyl acetate



NP-I titrated with ethyl acetate

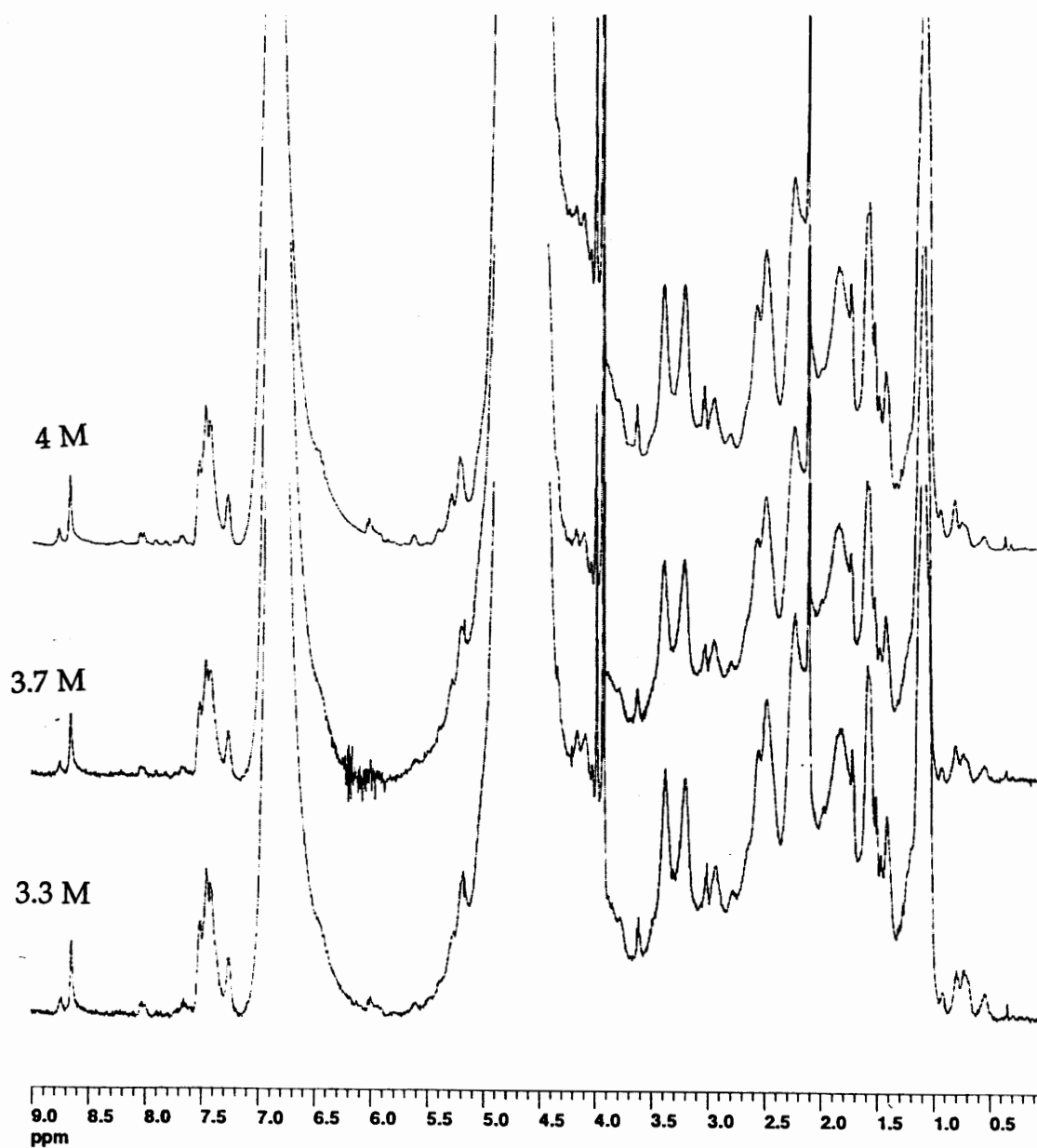


NP-I titrated with Gnd·DCI

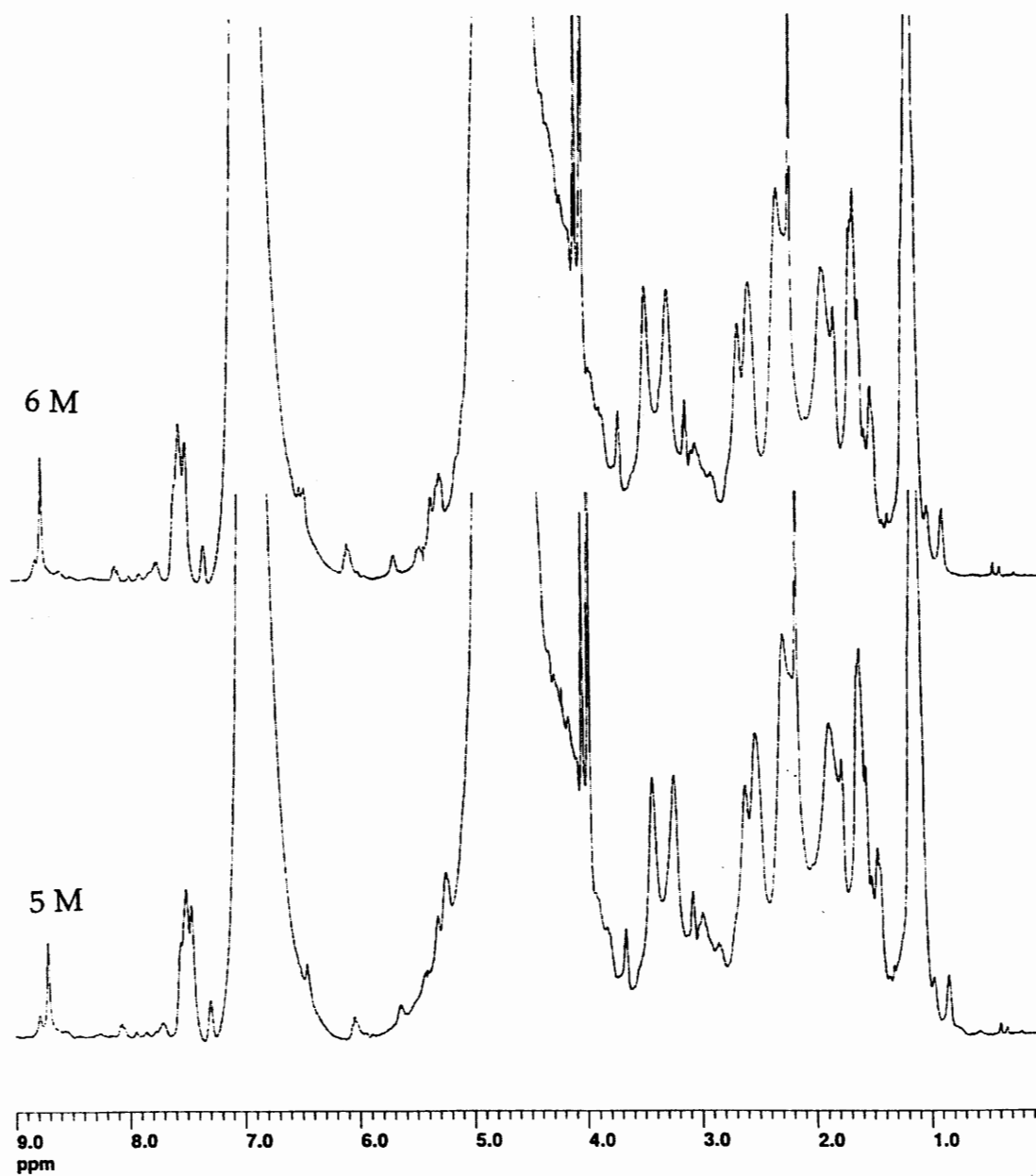


NP-I titrated with Gnd·DCI

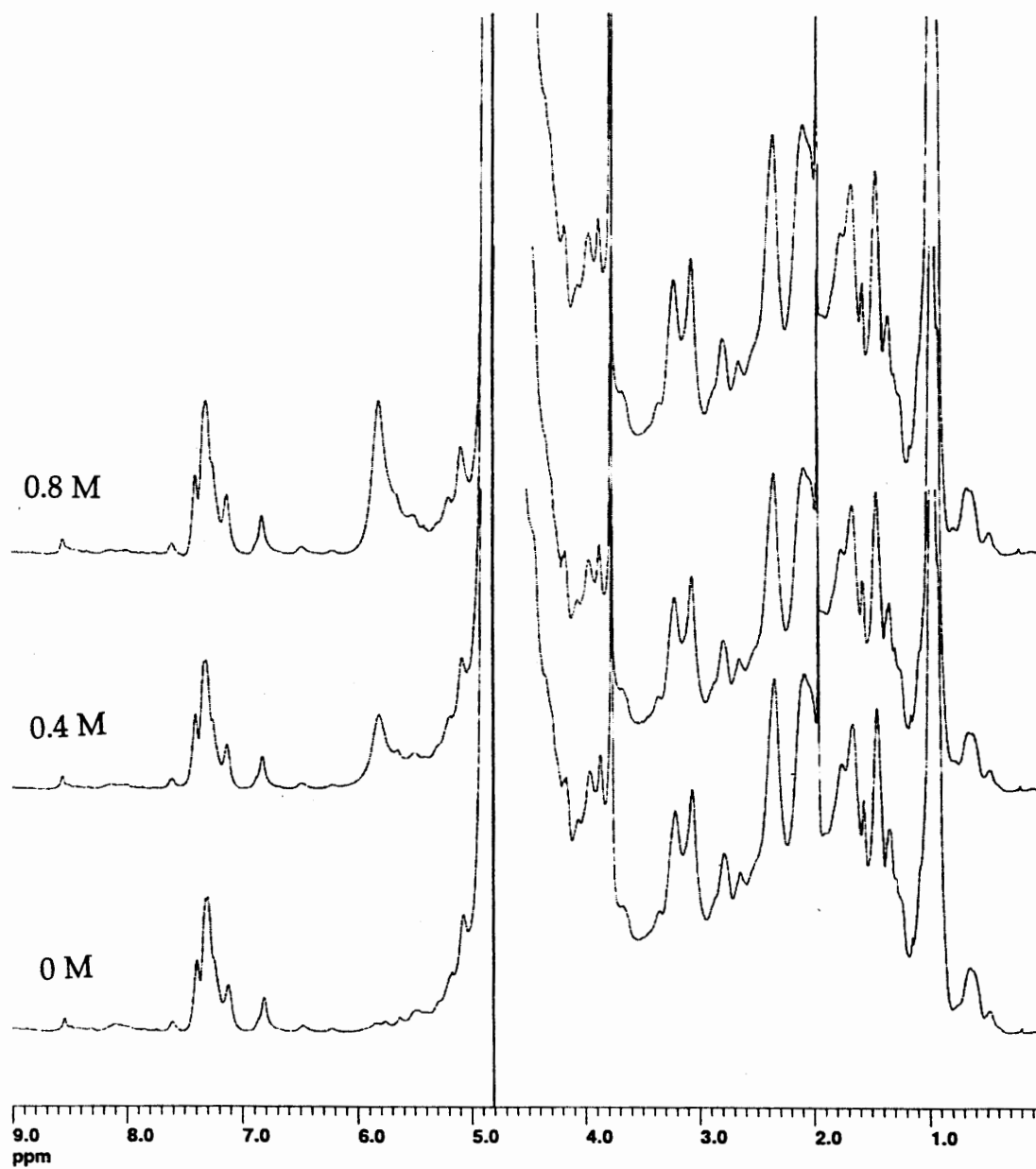




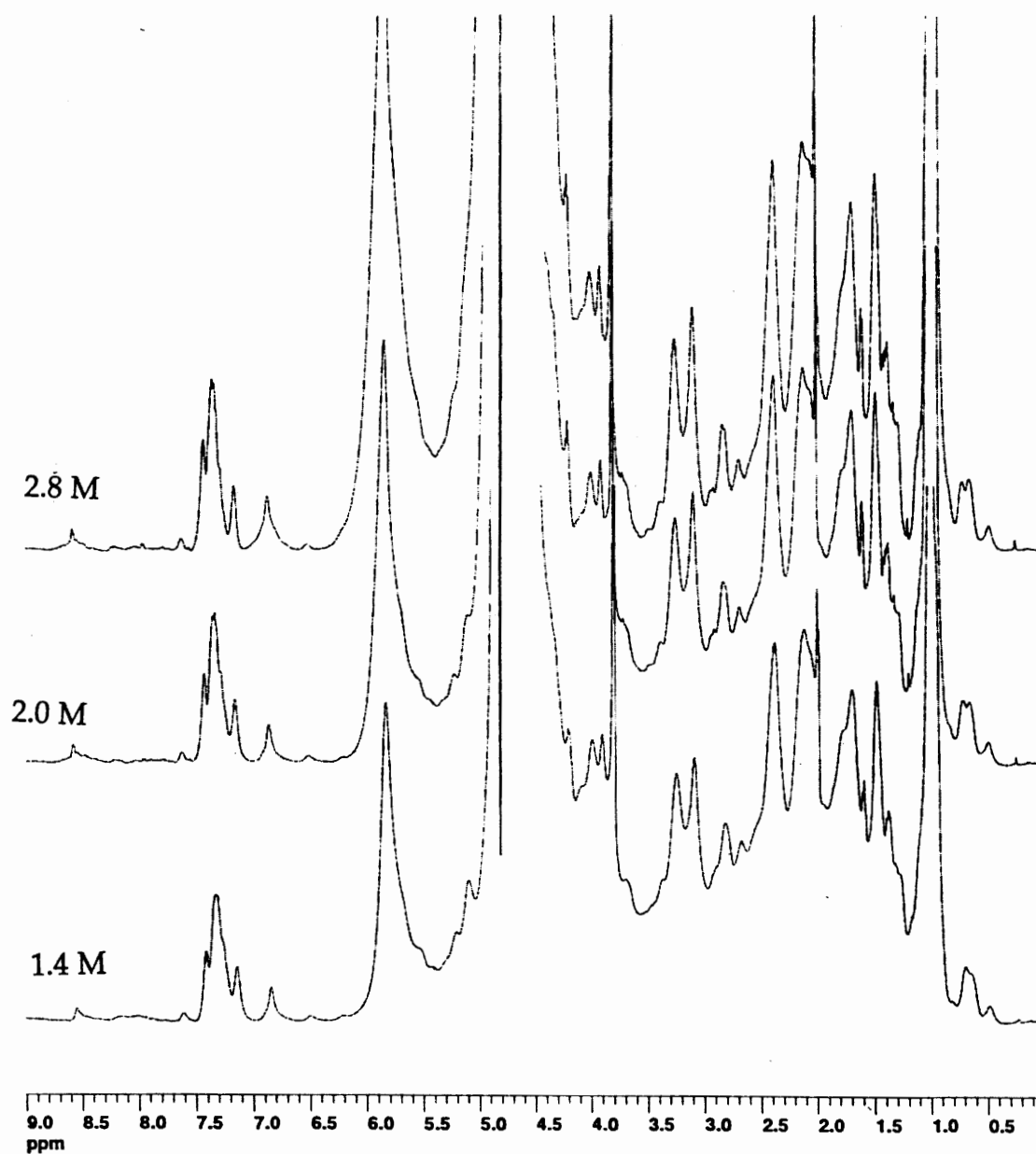
NP-I titrated with Gnd·DCI



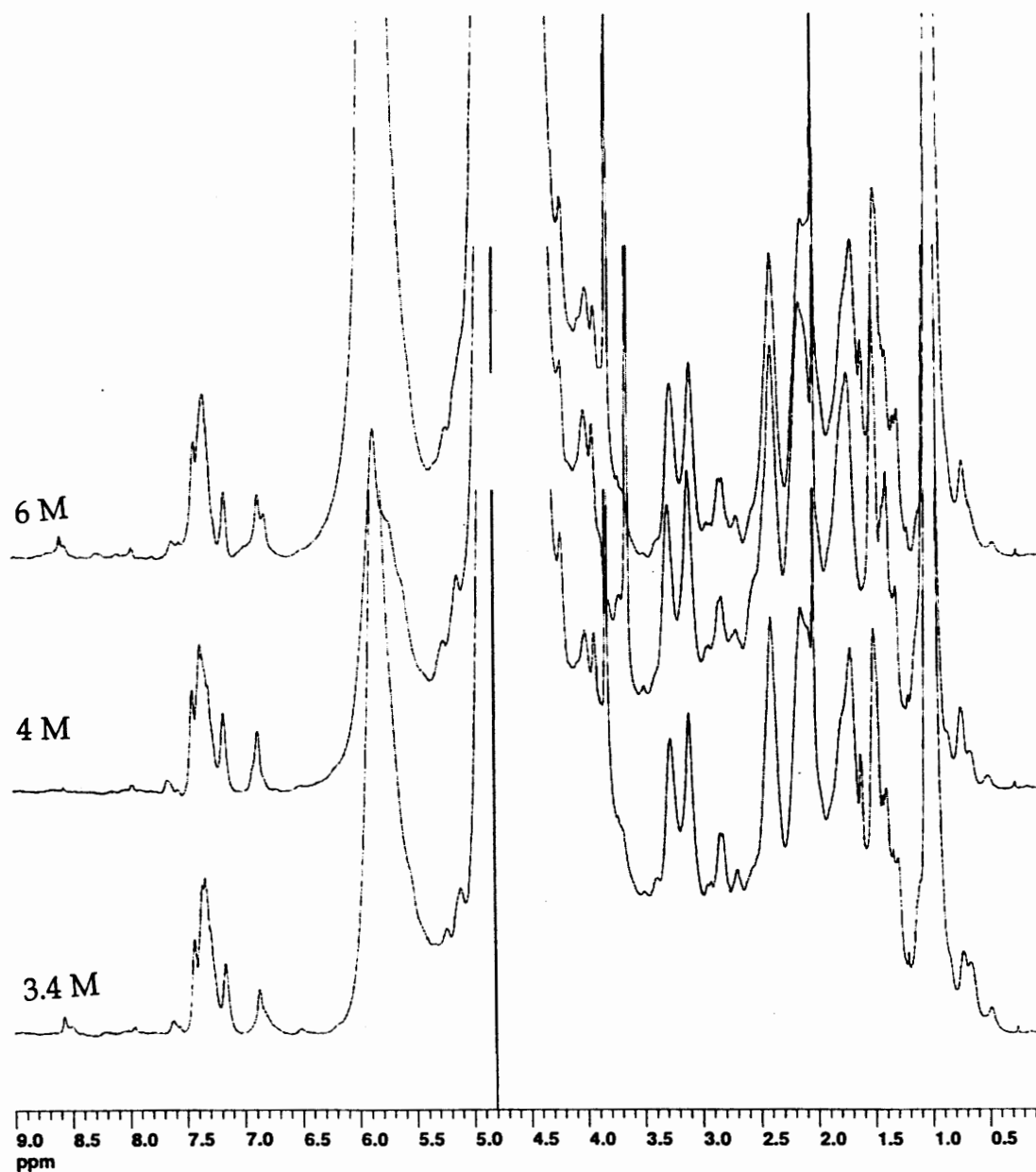
NP-I titrated with Gnd·DCI



NP-I titrated with urea



NP-I titrated with urea



NP-I titrated with urea